



Emma Holmlund

Mother-Infant Antibodies to Pneumococcal Polysaccharides and Proteins

Transfer and Persistence of Maternal Antibodies and Development of
Vaccine- Induced and Naturally Acquired Antibodies in Infants



RESEARCH 64

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ACADEMIC DISSERTATION

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ABSTRACT

Emma Holmlund. Mother-Infant Antibodies to Pneumococcal Polysaccharides and Proteins. Transfer and Persistence of Maternal Antibodies and Development of Vaccine-Induced and Naturally Acquired Antibodies in Infants. National Institute for Health and Welfare (THL). Research 64. 158 pages. Helsinki, Finland 2011. ISBN 978-952-245-487-4 (printed); ISBN 978-952-245-488-1 (pdf)

Symptomless nasopharyngeal carriage of *Streptococcus pneumoniae* (pneumococcus) is very common in young children. Occasionally, this carriage proceeds into mild mucosal diseases such as sinusitis or acute otitis media, or into serious life-threatening diseases such as pneumonia, sepsis or meningitis. Each year, up to one million children worldwide die of pneumococcal pneumonia, sepsis or meningitis. Especially in low-income countries, invasive pneumococcal disease (IPD) is a leading health problem in infants; 75% of all IPD cases occur before one year of age. This stresses the need for increased protection against pneumococcus in infancy. Anti-pneumococcal antibodies form an important component in the defence against pneumococcal infection. Maternal immunisation and early infant immunisation are two possible ways by which potentially protective antibody concentrations against pneumococci could be achieved in early infancy. Limited immunogenicity of the pneumococcal polysaccharide vaccine in infants and the limited serotype coverage of the pneumococcal conjugate vaccines have stimulated interest in pneumococcal protein vaccines. Pneumococcal protein antigens are immunogenic already in young children and have the potential to provide protection against pneumococci, regardless of serotype.

The aim of this thesis is to increase the knowledge related to antibody-mediated protection against pneumococcal disease in infants and young children. More specifically, we investigated the transfer of naturally acquired maternal anti-protein antibodies from mothers to their infants, the persistence of vaccine-induced maternal anti-polysaccharide antibodies as well as naturally acquired maternal anti-protein antibodies in the infants, the immunogenicity of the 23-valent pneumococcal polysaccharide vaccine (PPV) in infants and the response of the children to a second dose of PPV at three years of age. We also investigated the development of naturally acquired antibodies to certain pneumococcal protein antigens in relation to culture-confirmed pneumococcal carriage in infants.

The maternal immunisation study (MATER) was conducted in 1994–1998 in the Philippines. The serum samples collected from the mothers, the umbilical cords and the infants at 7 weeks, 14 weeks, 17 weeks, 22 weeks, 9 months and 10 months were

used to determine the antibody concentrations to pneumococcal serotypes 1, 5, 6B, 14, 18C and 19F, as well as to the pneumococcal proteins PspA, PsaA, Ply, PspC, PhtD, PhtDC and LytC by the enzyme immunoassay (EIA). We found that the naturally acquired maternal anti-protein antibodies were transferred from the mothers to the infants. The concentrations of maternal antibodies to serotypes 6B, 14, 18C and 19F were significantly higher for 4–5 months after birth in infants of immunised mothers compared to infants of unimmunised mothers. In infants, antibody responses to serotypes 1 and 5, but not to serotypes 6B, 14, 18C or 19F, were detected after immunisation with PPV at seven or 14 weeks of age. At three years of age, the children responded well to the second dose of PPV, suggesting that maternal and early infant immunisations might not induce hyporesponsiveness to polysaccharide antigens after subsequent immunisations. The naturally acquired maternal anti-protein antibodies persisted in the infants for 4–5 months after birth. The production of naturally induced antibodies to PspA, Ply, PspC, PhtD, PhtDC and LytC in infants started at 4–5 months of age in response to pneumococcal carriage, whereas the production of anti-PsaA antibodies started already after seven weeks of age.

The findings of the present study on the anti-polysaccharide antibodies in mothers and infants confirm previously obtained results and add to the global knowledge of responses to PPV in young children. Immunising pregnant women with PPV provides the infants with increased concentrations of pneumococcal polysaccharide antibodies. Of the six serotypes examined, serotypes 1 and 5 were immunogenic already in infants. The anti-protein antibody findings provide useful information for the development of pneumococcal protein vaccines. All six proteins studied were immunogenic in infancy and the development of anti-protein antibodies started early in life. Since anti-protein antibodies are transferred from the mothers to the infants, both pregnant women and infants could be immunised with future pneumococcal protein vaccines in order to achieve potential protection of the infant against pneumococcal disease during early life.

Keywords: *Pneumococcus*, maternal antibodies, early infant immunisation, hyporesponsiveness, development of antibodies to pneumococcal proteins, pneumococcal carriage

TIIVISTELMÄ

Emma Holmlund. Mother-Infant Antibodies to Pneumococcal Polysaccharides and Proteins. Transfer and Persistence of Maternal Antibodies and Development of Vaccine-Induced and Naturally Acquired Antibodies in Infants. [Äidin ja imeväisen pneumokokkipolysakkaridi- ja proteiinivasta-aineet. Äidin vasta-aineiden siirtyminen ja säilyminen sekä rokotuksen aikaansaama ja luonnollisesti syntynyt vasta-ainekehitys imeväisissä.]. Terveiden ja hyvinvoinnin laitos (THL). Tutkimus 64. 158 sivua. Helsinki, Finland 2011. ISBN 978-952-245-487-4 (painettu); ISBN 978-952-245-488-1 (pdf)

Pikkulapsien oireeton *Streptococcus pneumoniae* –bakteerin eli pneumokokin nenänielukantajuus on hyvin yleistä. Joskus pneumokokin nenänielukantajuus voi edetä lieväksi limakalvoihin rajoittuvaksi tulehdukseksi kuten esimerkiksi poskiontelon- tai välikorvantulehdukseksi, toisinaan vakavaksi henkeä uhkaavaksi taudiksi kuten keuhkokuumeeksi, verenmyrkytykseksi tai aivokalvontulehdukseksi. Maailmassa joka vuosi jopa miljoona lasta kuolee pneumokokin aiheuttamaan keuhkokuumeeseen, verenmyrkytykseen tai aivokalvontulehdukseen. Varsinkin matalan tulotason maissa invasiiviset pneumokokkitaudit ovat imeväisikäisten merkittävä ongelma; 75% kaikista pneumokokin aiheuttamista invasiivisista taudeista esiintyy alle vuoden ikäisissä. Tämä korostaa tarvetta suojata imeväisikäisiä paremmin pneumokokkitauteja vastaan. Pneumokokkivasta-aineet ovat tärkeä osa elimistömme puolustusmekanismeja pneumokokki-infektioita vastaan. Raskaana olevien äitien sekä imeväisten varhainen rokottaminen ovat mahdollisia keinoja, joilla imeväisiä todennäköisesti suojaavia vasta-ainetasoja pneumokokkia vastaan voitaisiin lisätä. Pneumokokkipolysakkaridirokotteiden vähäinen immunogeenisuus imeväisissä sekä pneumokokkikonjugaattirokotteiden serotyyppikattavuuden rajoittuneisuus ovat herättäneet kiinnostusta pneumokokki-proteiinirokotteita kohtaan. Pneumokokin proteiiniantigeenit ovat immunogeenisiä jo nuorissa lapsissa. Niillä on mahdollisuus tuottaa serotyypistä riippumatonta suojaa pneumokokkia vastaan.

Tämän väitöskirjatutkimuksen tavoite on lisätä tietoa vasta-aineiden tuottamasta suojasta pneumokokkitauteja vastaan sekä imeväisissä että pikkulapsissa. Erityisesti tutkimme miten luonnollisesti syntyneet proteiinivasta-aineet siirtyvät äideistä lapsilleen, miten rokotteen aikaansaamat äitien polysakkaridivasta-aineet sekä äitien luonnollisesti syntyneet proteiinivasta-aineet säilyvät lapsissa, kuinka immuno-geeninen 23-valenttinen pneumokokkipolysakkaridirokote (PPV) on imeväisissä sekä miten lapset muodostavat vasta-ainevasteita toiselle PPV annokselle kolmen

vuoden iässä. Tutkimme myös luonnollisten vasta-aineiden kehittymistä muutamille proteiiniantigeeneille suhteessa imeväisten pneumokokki-kantajuteen.

Rokotetutkimus MATER, jossa äitejä ja heidän vastasyntyneitä lapsiaan rokotettiin, tehtiin Filippiineillä vuosina 1994-1998. Seeruminäytteet jotka kerättiin äideistä, napanuorista sekä imeväisistä 7, 14, 17 ja 22 viikon iässä sekä 9 ja 10 kuukauden iässä, analysoitiin entsyymi-immunologisin menetelmin (EIA). Vasta-ainepitoisuudet määritettiin pneumokokkiserotyypille 1, 5, 6B, 14, 18C ja 19F sekä proteiineille PspA, PsaA, Ply, PspC, PhtD, PhtDC ja LytC. Totesimme, että äideistä tulleet luonnollisesti syntyneet proteiinivasta-aineet siirtyvät äidistä lapseen. Äidin muodostamat 6B, 14, 18C ja 19F vasta-aineet olivat 4-5 kuukautta syntymän jälkeen merkitsevästi korkeammat lapsissa, joiden äitejä oli rokotettu verrattuna lapsiin joiden äitejä ei ollut rokotettu. Lapsissa rokotevasteita nähtiin serotyypille 1 ja 5, mutta ei serotyypille 6B, 14, 18C tai 19F PPV rokotuksen jälkeen, joka oli annettu lapsille 7 tai 14 viikon iässä. Kolmen vuoden iässä lasten vasta-ainevaste toiselle rokoteannokselle oli hyvä, mikä viittaa siihen, että äitien ja lasten varhainen rokottaminen polysakkaridirokotteella ei mahdollisesti aiheuttaisi niin sanottua hyporesponsiivisuutta polysakkaridiantigeeneille toistuviin rokotuksiin liittyen. Äideistä tulleet luonnollisesti syntyneet proteiinivasta-aineet säilyivät lapsissa 4-5 kuukautta syntymän jälkeen. Luonnollisten vasta-aineiden tuotanto proteiineille PspA, Ply, PspC, PhtD, PhtDC ja LytC alkoi 4-5 kuukauden iässä pneumokokkikantajuuden ansiosta kun taas PsaA-vasta-ainetuotanto alkoi jo 7. ikäviikon jälkeen.

Tämän tutkimuksen tulokset äitien ja lasten polysakkaridivasta-ainevasteista vahvistavat aikaisempia löydöksiä ja lisäävät maailmanlaajuisia tietoutta vasteista polysakkaridirokotteille nuorissa lapsissa. Raskaana olevien naisten rokottaminen PPV:llä varustaa imeväisiä lisääntyneillä polysakkaridivasta-ainepitoisuuksilla. Kuudesta tutkitusta pneumokokin serotyypistä serotyypit 1 ja 5 olivat immunogeenisiä jo imeväisissä. Proteiinivasta-ainelöydökset tuovat hyödyllistä tietoa pneumokokkirokotekehitystä varten. Kaikki kuusi tutkittua proteiiniantigeenia olivat immunogeenisiä imeväisissä. Proteiinivasta-aineiden tuotanto alkoi jo varhain elämässä. Kun proteiinivasta-aineiden on osoitettu siirtyvän äidistä lapseen, raskaana olevia naisia sekä imeväisiä voitaisiin rokottaa tulevaisuuden proteiinirokotteilla, jotta imeväiset saisivat parhaan mahdollisen suojan pneumokokkitauteja vastaan elämänsä varhaisessa vaiheessa.

Avainsanat: Pneumokokki, äitien vasta-aineet, imeväisen varhainen rokottaminen, hyporesponsiivisuus, luonnollisten vasta-aineiden kehitys, pneumokokkinenänielukantajuus

SAMMANDRAG

Emma Holmlund. Mother-Infant Antibodies to Pneumococcal Polysaccharides and Proteins. Transfer and Persistence of Maternal Antibodies and Development of Vaccine-Induced and Naturally Acquired Antibodies in Infants. [Moderns och spädbarnets antikroppar mot pneumokockens polysackarider och proteiner. Överföring av moderns antikroppar och deras beständighet samt vaccinflamkallade och naturligt uppkomna antikroppars utveckling hos spädbarn.]. Institutet för hälsa och välfärd (THL). Forskning 64. 158 sidor. Helsingfors, Finland 2011. ISBN 978-952-245-487-4 (tryckt); ISBN 978-952-245-488-1 (pdf)

Det är mycket vanligt att småbarn bär på *Streptococcus pneumoniae* (pneumokocker) i näs-svalgrummet utan att ha symptom. Ibland kan pneumokocken ändå förorsaka lindriga infektioner på slemhinnorna såsom bihåle- eller öroninflammationer eller livshotande infektioner som till exempel lunginflammation, blodförgiftning eller hjärnhinneinflammation. Årligen dör upp till 1 miljon barn runt om i världen till följd av lunginflammation, blodförgiftning eller hjärnhinneinflammation som förorsakats av pneumokocker. Speciellt i låginkomstländer är invasiva pneumokocksjukdomar ett betydande hälsoproblem hos spädbarn; 75 procent av alla invasiva pneumokocksjukdomar förekommer hos barn under 1 år. Det här framhäver behovet av skydd mot pneumokockinfektioner hos småbarn. Antikropparna mot pneumokockerna utgör en viktig del av vårt försvar mot pneumokockinfektioner. Att vaccinera väntande mammor eller spädbarn i tidig ålder är två möjliga sätt på vilka potentiella skyddande antikroppskoncentrationer kan uppnås i tidig ålder. Polysackaridvaccinernas begränsade immunogenicitet hos spädbarn och konjugatvaccinernas begränsade serotypäckning har väckt ett intresse för proteinbaserade pneumokockvacciner. Pneumokockens proteinantigener är immunogena redan hos spädbarn och har potential att skydda mot pneumokocker oberoende av serotyp.

Syftet med denna avhandling är att bidra med kunskap om ett antikroppbaserat skydd mot sådana sjukdomar hos spädbarn och småbarn som förorsakas av pneumokocker. Undersökningen fokuserade på hur naturligt uppkomna proteinantikroppar överförs från mammor till deras barn, hur länge de genom vaccinering framkallade polysackaridantikroppar och de naturligt uppkomna proteinantikropparna som överförts från mamman finns kvar hos spädbarnen, det 23-valenta polysackaridvaccinets (PPV) immunogenicitet hos spädbarn och vaccinresponsen hos 3-åriga barn efter en andra dos av PPV. Undersökningen fokuserade dessutom på den naturliga utvecklingen av antikroppar mot vissa

pneumokockproteinantigener i förhållande till om barnet var bärare av pneumokocker eller inte.

Vaccinundersökningen MATER, där mammor och deras barn vaccinerades, utfördes på Filippinerna åren 1994-1998. Serumproverna som togs av mammorna, från navelsträngarna och av barnen vid 7, 14, 17 och 22 veckors ålder samt vid 9 och 10 månaders ålder användes för att med en enzymimmunologisk metod (EIA) bestämma koncentrationen av pneumokockantikroppar mot serotyperna 1, 5, 6B, 14, 18C och 19F, samt mot pneumokockproteinerna PspA, PsaA, Ply, PspC, PhtD, PhtDC och LytC. Vårt forskningsteam konstaterade att de naturligt framkallade proteinantikropparna överförs från mamman till barnet. Koncentrationen av antikroppar mot serotyperna 6B, 14, 18C och 19F var under 4-5 månader efter födseln betydligt högre hos barn vars mammor vaccinerats med PPV jämfört med barn vars mammor inte vaccinerats. Efter vaccineringen av spädbarnen vid 7 eller 14 veckors ålder konstaterades en ökning av antikroppskoncentrationen mot serotyperna 1 och 5, men inte mot 6B, 14, 18C eller 19F. Responsen till följd av den andra PPV-dosen var god vid 3 års ålder, vilket tyder på att vaccineringen av mammor och spädbarn med polysackaridantigener möjligen inte förorsakar hyporespons hos barn efter upprepade vaccinationer. De naturligt uppkomna proteinantikroppar som hade överförts från mammorna fanns kvar hos barnen i 4-5 månader efter födseln. Den naturliga produktionen av antikroppar mot proteinerna PspA, Ply, PspC, PhtD, PhtDC och LytC började vid 4-5 månaders ålder till följd av att barnet bar pneumokocker i näs-svalgrummet, medan produktionen av PsaA-antikroppar började redan efter 7 veckors ålder.

Den här undersökningens resultat gällande polysackaridantikroppar hos mammor och spädbarn bekräftar tidigare resultat och ökar den globala kunskapen om responsen hos barn till följd av vaccinering med PPV. Genom att vaccinera väntande mammor med PPV får barnen förhöjda koncentrationer av pneumokock-polysackaridantikroppar. Av de 6 undersökta serotyperna var serotyperna 1 och 5 immunogena hos spädbarn. Resultaten gällande proteinantikropparna bidrar med användbar information för utvecklingen av proteinvacciner mot pneumokocker. Alla undersökningens 6 proteiner var immunogena hos spädbarn och produktionen av proteinantikroppar började redan i tidig ålder. Eftersom proteinantikroppar överförs från mamman till barnet, kunde både gravida kvinnor och spädbarn vaccineras med framtida pneumokock-proteinvacciner för att barnet redan i tidig ålder ska få ett potentiellt skydd mot pneumokocksjukdomar.

Nyckelord: pneumokocker, antikroppar från mamman, vaccinering av spädbarn i tidig ålder, hyporespons, pneumokockproteinantikropparnas utveckling, bära pneumokocker i näs-svalgrummet.

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- I Holmlund E, Nohynek H, Quiambao B, Ollgren J, Käyhty H. Mother-infant vaccination with pneumococcal polysaccharide vaccine: Persistence of maternal antibodies and responses of infants to vaccination. *Vaccine* 2011;29(28):4565-75.
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ABBREVIATIONS

ACIP	advisory committee on immunisation practices
AOM	acute otitis media
ARI	acute respiratory tract infection
BCG	Bacille Calmette-Guerin vaccine against tuberculosis
CI	confidence interval
Control 1 infants	no PPV given to mothers, PPV given to infants at 7 wk of age
Control 2 infants	no PPV given to mothers nor to infants
CDR	clade defining region
CoDRs	complementarity determining regions
CPS	cell wall polysaccharide
CR	complement receptor
CPR	C-reactive protein
DC	dendritic cell
C-term	carboxyterminal end
DTwP	diphtheria, tetanus and whole cell pertussis vaccine
ECM	extracellular matrix
EIA	enzyme immunoassay
22F EIA	EIA with 22F polysaccharide neutralization
non-22F EIA	EIA without 22F polysaccharide neutralization
Eno	enolase
EPI	extended program of immunisation
Fab	antigen-binding fragment
Fc	crystallisable non-antigen binding fragment
FcγR	IgG binding receptor
GMC	geometric mean concentration
HBV	hepatitis B vaccine
Hib	<i>Haemophilus influenzae</i> type b
Hyl	hyaluronan lyase
Ig	immunoglobulin
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IPD	invasive pneumococcal disease
LytA, B, C	autolysin enzyme A, B, C
MATER	the maternal immunisation study
MHCII	major histocompatibility complex class II
MLST	multilocus sequence typing

NanA	neuraminidase A
N-term	aminoterminal end
OPV	oral polio vaccine
PAFr	platelet-activating factor receptor
PAMP	pathogen-associated molecular patterns
PavA and B	pneumococcal adhesion and virulence factor A and B
PCCho	phosphorylcholine
PCR	polymerase chain reaction
PCV	pneumococcal conjugate vaccine
PCV7	7-valent pneumococcal conjugate vaccine
PCV10	10-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
PfbA	plasmin- and fibronectin-binding protein A
PhtD	Pneumococcal histidine triad protein D
PhtDC	C-terminal end of PhtD
pIgR	polymeric immunoglobulin receptor
Ply	pneumolysin or pneumolysoid
Pnc	pneumococcus
Pnc+	infants with previous or current pneumococcal carriage
Pnc-	infants with no detected pneumococcal carriage
Pneumococcus	<i>Streptococcus pneumoniae</i>
PpmA	putative proteinase maturation protein
PS	polysaccharide
PsaA	pneumococcal surface adhesin A
PspA	pneumococcal surface protein A
PspC	pneumococcal surface protein C
pIgR	polymeric immunoglobulin receptor
PPV	23-valent pneumococcal polysaccharide vaccine
PPV1 infants	PPV given to mothers during pregnancy and to infants at 7 wk of age
PPV2 infants	PPV given to mothers during pregnancy and to infants at 17 wk of age
sIgA	secretory immunoglobulin A
SlrA	streptococcal lipoprotein rotamase A
TD	T-cell dependent
TI	T-cell independent
TNF- α	tumor necrosis factor α
TLR	Toll-like receptor
U/ml	units per millilitre
WHO	world health organisation
ZmpB	zinc metalloprotease B

1 INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is the leading cause of bacterial pneumonia, bacteraemia and meningitis in children less than five years of age worldwide. It has been estimated that up to one million children less than five years old die annually of pneumococcal diseases, mostly in low-income countries. Pneumococcus is able to colonise the human nasopharynx without causing symptoms in the host. By mechanisms and host pathogen interactions not fully understood, pneumococci cause limited mild mucosal infections in some hosts, while causing severe life-threatening invasive infections in others. Pneumococcus is a heterogeneous species and can be divided into more than 90 different serotypes based on the structure of its capsular polysaccharides.

Pneumococcal infections, irrespective of serotype, can be successfully treated with antibiotics, but the increasing resistance among pneumococci to antibiotics has highlighted the need for prevention, which can be achieved by vaccination. Currently, there are two types of licensed pneumococcal vaccines, the 23-valent pneumococcal polysaccharide vaccine (PPV) and the pneumococcal conjugate vaccines (PCVs). PPV is not immunogenic to all serotypes in children less than two years of age and the PCVs have limited serotype coverage. This has raised interest in a new type of vaccine, specifically the pneumococcal protein vaccines. The protein vaccines have the potential to protect against all serotypes and they are immunogenic already in infants. Since protection against pneumococcal infections early in life is essential, several prevention strategies should be considered, including maternal and early infant immunisation.

The purpose of this thesis is to increase the knowledge of antibody-mediated protection against pneumococcal disease in infants and young children. One of its specific aims is to investigate the transfer of naturally acquired maternal anti-protein antibodies to the infants and their persistence, as well as the persistence of vaccine-induced anti-polysaccharide maternal antibodies in the infants. Another aim is to investigate the immunogenicity of PPV in infants and the response to a second dose at three years of age. A further goal is to investigate the development of naturally acquired anti-protein antibodies to certain pneumococcal protein antigens and to relate the antibody development to pneumococcal nasopharyngeal carriage. This information will provide useful information for future vaccine development aiming at protecting infants and children. Maternal immunisation has become an important method of infant protection, as demonstrated by the successful maternal tetanus toxoid vaccination in low-income countries, and recently by maternal influenza vaccinations.

2 REVIEW OF THE LITERATURE

2.1 Pneumococcal infections

2.1.1 The bacterium

Pneumococcus was first isolated and described in 1881 by Louis Pasteur in France and a few months later by George Miller Sternberg in the United States (Henrichsen 1999). *Pneumococcus* is an extensively studied bacterium, which has significantly contributed to the basics of molecular biology and to the understanding that DNA is the carrier of genetic information (Avery et al. 1944), as well as to our understanding of bacterial infections, antibiotic resistance mechanisms (Lynch et al. 2009a) and the ability of polysaccharide and protein antigens to induce protective antibodies (Heidelberger et al. 1923; Austrian 1981b, a). *Pneumococcus* is a Gram-positive, facultative anaerobic bacterium that appears in pairs or short chains. The bacterium is surrounded by a polysaccharide capsule. Based on the differences in the structure of the capsule, pneumococci can be grouped into more than 90 immunologically distinct serotypes (Henrichsen 1995; Park et al. 2007; Bratcher et al. 2010; Calix et al. 2010). The currently used Danish nomenclature system groups the pneumococcal serotypes according to their chemical structure and antigenic characteristics. For example, serotypes 6A, 6B and 6C are serologically cross-reactive and are all grouped into the same serogroup, serogroup 6 (Lund et al. 1978; Park et al. 2007).

The conventional methods for identifying pneumococci are based on colony morphology, Gram-stain and optochin sensitivity (Lund et al. 1978). A more modern method is the polymerase chain reaction (PCR)-based technique, by which, e.g. different pneumococcal proteins may be identified (Salo et al. 1995; Park et al. 2010; Vernet et al. 2011). Rapid tests that identify pneumococcal antigens directly from the clinical samples are also available (Gisselsson-Solen et al. 2007). To identify the pneumococcal serotype, the gold standard method is the immunochemical quellung reaction based on capsular swelling (Henrichsen 1979). Recently, a new PCR-based method has been used to amplify the gene areas specific to capsular polysaccharides (Pai et al. 2006). Another new method that has not yet been widely used is a multiplex assay based on serotype-specific monoclonal antibodies (Yu et al. 2005). For global pneumococcal epidemiology, the molecular identification of pneumococcal strains by multilocus sequence typing (MLST) is employed (Enright et al. 1998).

2.1.2 *Pneumococcus* is a successful coloniser

The colonisation of the human nasopharynx is a dynamic process: Bacteria are acquired, eliminated and re-acquired many times during life (Ghaffar et al. 1999). The nasopharyngeal flora becomes established during the first year of life (Aniansson et al. 1992; Kononen et al. 2002; Hill et al. 2008). The mucosa of the nasopharynx becomes densely colonised by a broad variety of microorganisms (e.g. viridans streptococci, diphtheroids, pneumococci, non-typable *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus*), of which some might cause disease (Loda et al. 1975; Aniansson et al. 1992; Lankinen et al. 1994; Kononen et al. 2002; Konno et al. 2006; Jourdain et al. 2011). A viral infection might predispose the nasopharynx to pneumococcal colonisation events, by exposing receptors for pneumococcal adherence; this may eventually lead to pneumococcal disease (Peltola et al. 2004). The nasopharynx lies between the nose, the sinuses, the ears, the larynx and the lower respiratory tract and therefore the carried nasopharyngeal bacteria are a potential source of pathogens causing upper and lower respiratory tract infections.

Pneumococci are human-specific extracellular pathogens spread by droplet secretions or intimate contact (Musher 2003). The first step in infection is nasopharyngeal colonisation and asymptomatic pneumococcal carriage. The nasopharynx is a region highly exposed to oxygen, which makes colonisation for the catalase-negative pneumococci a challenge. The competition for free colonisation sites in the nasopharynx among different bacteria is hard, as well as the fight against the human immune defence mechanisms. Pneumococci, however, have many mechanisms to overcome these challenges and this makes them successful colonisers. To handle the high oxygen level, pneumococci do not use the amino acid cysteine in exported proteins, thus evading oxidation of the thiol groups of cysteine (Daniels et al. 2010b). To resist the human lysozyme enzymes, which are abundant in the mucus, pneumococci modify their peptidoglycan with the enzymes PgdA and Adr (Davis et al. 2008). To render their competitors in the nasopharynx more susceptible to complement-mediated phagocytosis, the pneumococcal neuraminidase NanA cleaves the terminal sialic acids of the competitors (Shakhnovich et al. 2002). To further outcompete the other inhabitants of the nasopharynx, pneumococci secrete antimicrobial peptides called bacteriocins (Lux et al. 2007). Additionally, pneumococci have enzymes capable of retrieving carbohydrates needed for growth from the glycans in the mucosa, as well as for exposing receptors for adherence (Kadioglu et al. 2008; Trappetti et al. 2009). Finally, pneumococci with a thin capsule (transparent pneumococci) have a number of adhesive properties, and are

more prone to form biofilms (Parker et al. 2009; Henriques-Normark et al. 2010). Bacteria growing in biofilms have increased resistance to innate and acquired immunity, as well as to antibiotics (Moscoso et al. 2006; Slinger et al. 2006; Murphy et al. 2009).

Although pneumococci are successful colonisers of the nasopharynx, not all people are colonised with pneumococci all the time. The incidence of pneumococcal carriage varies drastically between different age groups, populations and regions. By three months of age, 12% of Finnish infants carry pneumococci in their nasopharynx (Syrjänen et al. 2001), while almost 50% of infants in Bangladesh (Granat et al. 2007), about 80% of infants in the Gambia (Hill et al. 2008; Darboe et al. 2010) and all infants in Papua New Guinea (Montgomery et al. 1990) are pneumococcal carriers. The duration of pneumococcal carriage varies, but the reasons for this are poorly understood (Garcia-Rodriguez et al. 2002). At least the capsular serotype has been shown to correlate with the duration of carriage (Sleeman et al. 2006). Risk factors for pneumococcal carriage include young age, having older siblings and day care attendance (Principi et al. 1999; Leino et al. 2008; Sa-Leao et al. 2008). Transmission of pneumococci occurs from carriers to others most efficiently in close contacts, such as in families or day care centres (Givon-Lavi et al. 2002; Melegaro et al. 2004b; Erästö et al. 2010). In a recent Gambian study, a strong association was shown between maternal and infant serotype colonisation, but reducing only the mother-infant transmission would have a minimal effect on the infants' total carriage (Darboe et al. 2010).

2.1.3 Pneumococcal disease in infants and young children

Asymptomatic pneumococcal carriage may proceed to pneumococcal disease by mechanisms not fully understood. Pneumococci may spread from the nasopharynx to the sinuses, causing sinusitis, or to the middle ear, causing acute otitis media (AOM). Pneumococci cause 30–60% of all AOM cases worldwide (Rodgers et al. 2009). They may also spread to the lungs, the blood stream and after crossing the blood-brain barrier, to the central nervous system, causing pneumonia, sepsis and meningitis, respectively (Fig. 1). The most common form of severe pneumococcal disease is bacteraemic pneumonia followed by meningitis and then by sepsis (Mulholland 1999; Peltola 2001). When pneumococci spread to normally sterile parts of the body, the infections generated are collectively called invasive pneumococcal disease (IPD).

The general risk factors for IPD (diagnosed by positive blood or cerebrospinal fluid cultures) are age less than 2 or more than 65 years, ethnicity, alcoholism, underlying

medical conditions, viral infection and immunosuppression, including HIV infection (Watson et al. 2006; Watt et al. 2007; Bliss et al. 2008; Hjulster et al. 2008; Lynch et al. 2009b). In low-income countries, the risk factors include also malnutrition, low birth weight, non-exclusive breastfeeding during the first four months of life, lack of measles immunisation, indoor air pollution and crowding (Rudan et al. 2008).

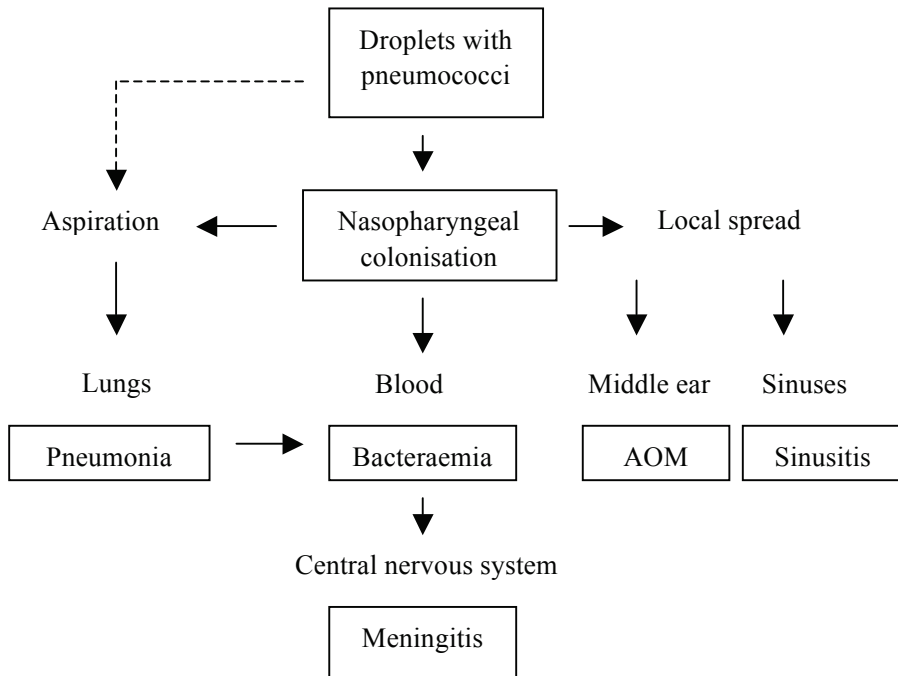


FIGURE 1. The main routes of pneumococcal spread (indicated by the solid lines) and the diseases caused. Figure modified from (Jambo et al. 2010).

Acute respiratory tract infections (ARIs) are an important public health problem in low-income countries (Garenne et al. 1992). They can be divided into upper respiratory tract infections (nose, sinuses, middle ear, larynx or pharynx) and lower respiratory tract infections (trachea, bronchi or lungs), of which the latter are more severe (Bellos et al. 2010). ARIs are caused by viruses or bacteria or both. The majority of ARI deaths are due to pneumonia, pneumococcus being the most common bacterial cause (Rudan et al. 2008). Based on four studies from low-income countries, it has been estimated that of all deaths due to ARI, 20% occurred in infants aged less than one month and 57% in infants aged 1–11 months (Garenne et al. 1992).

For a newborn child, the first few days and months of life involve the highest risk of death. Of the 130 million babies born each year, about 4 million die during the

neonatal period, i.e. in the first four weeks of life (WHO 2006). Most of the neonatal deaths occur in low-income countries, where the majority of the babies are born at home and most neonates also die at home. Therefore, there are very few data on the actual causes of death. It has been estimated that 36% of neonatal deaths are due to severe infections (26% due to sepsis/ pneumonia, 7% due to tetanus and 3% due to diarrhoea), although there are major variations between countries (Lawn et al. 2005). In a multicentre study that included sites from Ethiopia, the Gambia, Papua New Guinea and the Philippines, pneumococci accounted for 50% of all meningitis cases in infants aged one week to three months (The Who Young Infants Study Group 1999). In a more recent meta-analysis based on 63 studies published between 1980 and 2009, pneumococci were not a very common cause of IPD during the first week of life in low-income countries, but from the second to the fourth week they accounted for about 12% and from the fourth week to three months for 27% of all IPD cases (Zaidi et al. 2009). In the United States, the incidence of IPD peaks between 9 and 15 months of age and then reduces to approximately 50% of the peak at two years of age and to about 10–25% at three years of age (Lipsitch et al. 2005). In concordance, a study conducted in the Philippines reported that most IPD cases were seen in children less than one year of age (Lupisan et al. 2000). These studies indicate that pneumococcus is a notable pathogen during early childhood.

Different pneumococcal serotypes cause disease in different parts of the world. Most IPD cases are caused by a limited number of serotypes. The seven most common serotypes worldwide associated with IPD in children under five years of age are 1, 5, 6A, 6B, 14, 19F and 23F; of these, serotype 14 is the most important (Hausdorff 2007; Johnson et al. 2010). In the Philippines, serotype 1 is the most common cause of IPD, followed by serogroups 6, 14 and 23 in children less than five years of age (Lupisan et al. 2000). Together, these four serogroups accounted for about 90% of the IPD isolates (Lupisan et al. 2000). In Bangladesh, most IPD cases in children less than 5 years of age are caused by somewhat different serotypes: 2, 1, 14, 5, 7F, 45 and 12A (Saha et al. 2009).

2.2 Pneumococcal pathogenesis and virulence factors

To be capable of causing disease, pneumococci have to be able to invade the host and then resist and defeat the defence of the host. For this, pneumococci have several different properties, collectively called virulence factors. The understanding of these various pneumococcal virulence factors is important when developing new vaccines against pneumococci.

2.2.1 Structure and characteristics of the pneumococcal cell envelope

The pneumococcal cell envelope is formed by the cell membrane, the cell wall and the capsule (Fig. 2 and 3) (Skov Sorensen et al. 1988). The capsule is composed of capsular polysaccharides, which are anchored to the peptidoglycan of the cell wall (Skov Sørensen et al. 1990). The cell wall is composed of the peptidoglycan backbone, teichoic acids and lipoteichoic acids (Fig. 2) (Skov Sørensen et al. 1990; Fischer et al. 1993). The pneumococcal teichoic acid is unique among Gram-positive cell walls in that it contains phosphorylcholine (PCho) (Tomasz 1967; Fischer et al. 1993). This PCho-containing teichoic acid is called the cell wall polysaccharide (CPS). Antibodies to CPS are generated during an immune response, but these antibodies are not protective (Musher et al. 1990). The pneumococcal surface proteins are bound either to PCho or peptidoglycan structures of the cell wall, or to the lipids of the cell membrane (Rigden et al. 2003) (Fig. 2 and 3).

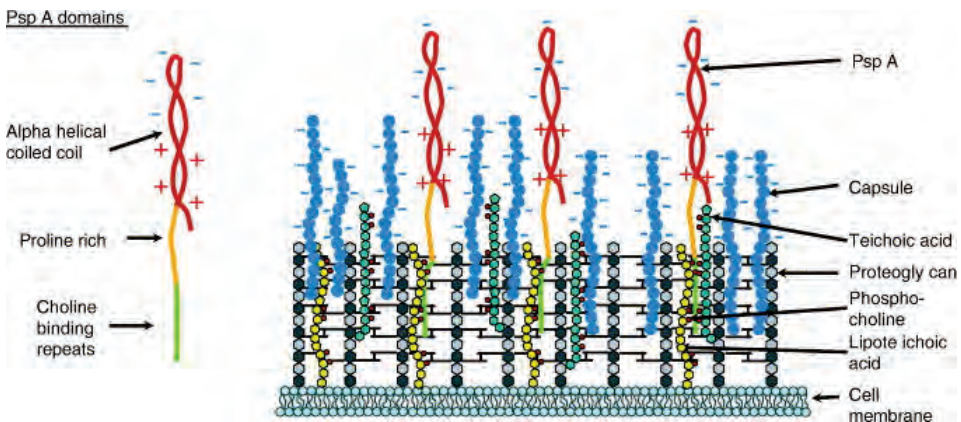


FIGURE 2. An illustration of the pneumococcal cell envelope; the cell membrane, the cell wall and the polysaccharide capsule. The cell wall is composed of the peptidoglycan backbone, the teichoic acids and the lipoteichoic acids. The PspA protein is anchored to the PCho and protrudes outside the capsule. Reprinted from (Daniels et al. 2006) with permission from Elsevier.

Pneumococcus is genetically a heterogeneous species that is able to quickly adapt to changing environments, such as various locations within the host. *Pneumococci* are able to spontaneously change the thickness of their capsule, between the transparent (thin) and opaque (dense) phenotype (Weiser et al. 1994; Hammerschmidt et al. 2005). This high-frequency phenomenon is called phase variation. The transparent variants are more efficient in colonising the nasopharynx (Weiser et al. 1994; Cundell et al. 1995b; Cundell et al. 1995a) and crossing the blood-brain barrier (Cundell et al. 1995b; Ring et al. 1998), whereas the opaque variants are more

virulent during a systemic infection (Kim et al. 1998). Transparent variants express more pneumococcal surface protein C (PspC) (Fig. 3), while opaque variants express more pneumococcal surface protein A (PspA) (Fig. 2 and 3) (Rosenow et al. 1997).

2.2.2 Adhesion to the host cells

Once pneumococci have entered the human upper respiratory tract, they need to adhere to host mucosal epithelial cells to cause colonisation. The colonisation process of pneumococci is not well understood. In a mouse model, the pneumococcal capsule has been shown to enhance colonisation (Nelson et al. 2007b). The negative charge of most capsular polysaccharides (Fig. 2) and the negative charge of the sialic acids on the mucus counteract the binding of pneumococci to the mucus, thus mediating the transfer of pneumococci to the epithelial surface (Nelson et al. 2007b). Once at the epithelial surface, phase variation probably occurs for stable colonisation. The pneumococcal enzyme neuraminidase A (NanA) (Fig. 3) removes sialic acid from the human glycoproteins on epithelial cells, thereby exposing receptors required for pneumococcal attachment (Andersson et al. 1983; King et al. 2006; Uchiyama et al. 2009).

Pneumococci attach to their receptors on the host epithelium by proteins called adhesins. PspC is a highly polymorphic protein that is able to adhere to resting lung cells and even more efficiently to cells activated by cytokines (Rosenow et al. 1997). In a mouse colonisation model, PspC⁻ mutants were significantly attenuated in their ability to colonise the mouse nasopharynx as compared to the parent strain (Balachandran et al. 2002). In addition, the pneumococcal surface adhesin A (PsaA) (Fig. 3) was earlier thought to be an adhesin (Sampson et al. 1994; Berry et al. 1996; Romero-Steiner et al. 2003; Romero-Steiner et al. 2006), but is nowadays suggested to play a regulatory role in adhesion through manganese transport (Johnston et al. 2004). The pneumococcal iron acquisition protein A (PiaA) and the pneumococcal iron uptake protein A (PiuA) (Fig. 3) are components of two pneumococcal iron transporters and are required for full virulence in a mouse model of infection (Brown et al. 2001b; Brown et al. 2001a). Some pneumococcal strains express pilus-like structures that have been shown to be important for colonisation (Barocchi et al. 2006; Nelson et al. 2007a). In addition, the pneumococcal protein putative proteinase maturation protein A (PpmA) and streptococcal lipoprotein rotamase A (SlrA) (Hermans et al. 2006; Cron et al. 2009), as well as the autolysin enzymes LytB and LytC (Gosink et al. 2000), have been shown to contribute to pneumococcal colonisation (Gosink et al. 2000; Hermans et al. 2006; Cron et al. 2009), even though these proteins are not actual adhesins. The intracellular toxin

pneumolysin (Ply) (Fig. 3) is not an adhesin either, but may play an important role in regulating nasopharyngeal immunity (Zhang et al. 2007; Lu et al. 2008; Dogan et al. 2011).

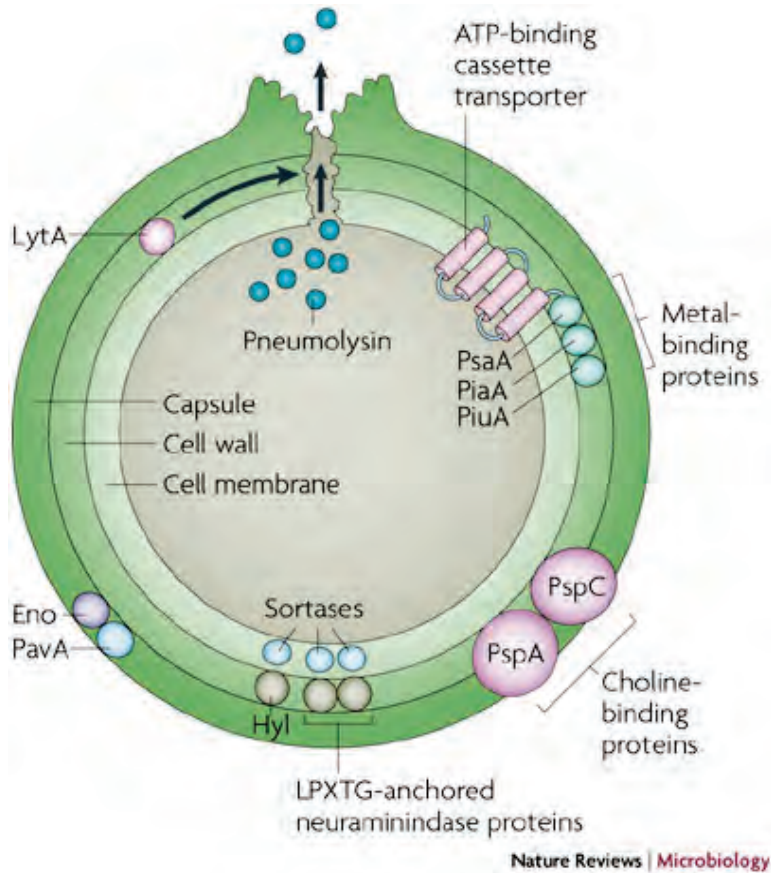


FIGURE 3. Essential pneumococcal virulence factors include the capsule; the cell wall; pneumococcal surface proteins A (PspA) and C (PspC); the neuraminidase proteins; hyaluronate lyase (Hyl); pneumococcal adhesion and virulence factor A (PavA); enolase (Eno); pneumolysin; autolysin A (LytA); and the metal-binding proteins pneumococcal surface adhesin A (PsaA), pneumococcal iron acquisition protein A (PiaA) and pneumococcal iron uptake protein A (PiuA). Reproduced with permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Kadioglu et al. 2008).

Secretory IgA1 bound to the pneumococcal surface mediates agglutination of the organism, thereby inhibiting pneumococcal attachment to the host tissues (Fasching et al. 2007). To circumvent this inhibition, pneumococci produce an IgA1 protease that is able to cleave bound IgA1, thereby increasing pneumococcal colonisation

(Weiser et al. 2003). These IgA1 proteases are thought to be important for the ability of pneumococci to colonise the nasopharynx, even in the presence of specific sIgA (Fasching et al. 2007).

2.2.3 Invasion and infection of the host

Pneumococci express many enzymes and proteins that are important for pneumococcal invasion. Hyaluronate lyase (Hyl) (Fig. 3) is an essential virulence protein that breaks down hyaluronan, an important constituent of the host connective tissue (Jedrzejewski et al. 2002). Enolase (Eno) (Fig. 3) is able to bind to the human enzyme plasmin and use it for the degradation of and migration through the host extracellular matrix (ECM) (Eberhard et al. 1999; Bergmann et al. 2005). The pneumococcal adherence and virulence factors A and B (PavA, PavB) (Fig. 3) have been shown to contribute to pneumococcal virulence by binding to the ECM protein fibronectin (Holmes et al. 2001; Jensch et al. 2010), which enables pneumococci to invade the epithelium (van der Flier et al. 1995). The plasmin- and fibronectin-binding protein A (PfbA) is important in adhesion and invasion of lung and laryngeal epithelial cells (Yamaguchi et al. 2008). Pneumococci are able to translocate across the mucosal barrier to the underlying tissues by binding to the polymeric Ig receptor (pIgR) (Zhang et al. 2000). PspC binds to the pIgR and pneumococci translocate by reverse transcytosis along with the receptor to the luminal side of the cell.

The major pneumococcal cell wall hydrolase, LytA (Fig. 3), releases small fragments of the pneumococcal cell wall, which induces macrophages to release proinflammatory cytokines (e.g. tumour necrosis factor α (TNF- α), interleukin (IL) - 1, IL-6) (Tuomanen et al. 1986). This triggers a network of additional inflammatory mediators and leads to the intense inflammatory reaction that accompanies pneumococcal infection (Tuomanen et al. 1987; Bruyn et al. 1992). In addition, PspC and the zinc metalloprotease B (ZmpB) have been shown to induce the expression of proinflammatory molecules, hence inducing pulmonary inflammation (Murdoch et al. 2002; Blue et al. 2003). In a recent study, Ply was also found to be a major factor responsible for the induction of inflammation (Yoo et al. 2010).

The induction of inflammation changes the type and number of receptors present on target epithelial and endothelial cells. One of the upregulated receptors is the platelet-activating-factor receptor (PAFr). Pneumococci are able to attach to the PAFr by PCho of the cell wall (Cundell et al. 1995b; Ring et al. 1998). PAFr has been shown to serve as a gateway for pneumococcal invasion of human tissue,

because pneumococci adherent to this receptor are able to translocate across the human tissue (Ring et al. 1998; Radin et al. 2005).

Ply is an intracellular toxin that has to be released from the bacterium to be able to mediate its virulence functions (Walker et al. 1987; Lock et al. 1992; Mitchell et al. 1997; Guiral et al. 2005; Price et al. 2009). By binding to the cell membrane cholesterol, Ply is capable of attaching to the cell membrane of a host cell (Walker et al. 1987). After insertion to the host cell membrane, Ply monomers form a transmembrane pore consisting of 30–50 monomeric units, which results in killing of the host cell through lysis (Morgan et al. 1995; Tilley et al. 2005). The damage caused to the host cells may assist pneumococci in invading tissue. Murine studies conducted with Ply⁻ mutants have proved the importance of Ply as a pneumococcal virulence factor: Ply⁻ mutant pneumococci caused milder forms of meningitis and they did not spread as efficiently to the bloodstream (Orihuela et al. 2004; Hirst et al. 2008).

2.2.4 Viral infections and pneumococcal invasion

The great majority of respiratory infections are of viral origin. Approximately 10–50% of patients with a viral respiratory tract infection develop a secondary bacterial infection, including AOM, sinusitis or pneumonia (Peltola et al. 2008). The mechanisms for the viral-bacterial synergy leading to the secondary bacterial infections are still poorly understood. The viral neuraminidases may contribute to the association seen between viral respiratory infections and pneumococcal infections by removing sialic acids from the host cell glycoconjugates, thereby exposing receptors for pneumococcal adherence (Peltola et al. 2004; Trappetti et al. 2009). In adherence experiments, rhinovirus-14 infection has been shown to stimulate pneumococcal adhesion to airway epithelial cells due to increases in PAFr (Ishizuka et al. 2003). Influenza infection increases the susceptibility of the host to pneumococcal infection through the suppression of neutrophil function and enhanced cytokine production (McNamee et al. 2006). In mice, immunisation with PspA was shown to mitigate the early secondary pneumococcal lung infections related to influenza virus infection (King et al. 2009). Pulmonary interferon- γ produced by the T-cells in response to influenza infection was shown to inhibit the bacterial clearance from the lungs by alveolar macrophages in mice (Sun et al. 2008). Interferon- γ has also been shown to increase the expression of the pIgR (Ackermann et al. 1999), which pneumococci are able to utilise for translocation across the mucosal barrier to the underlying tissues (Zhang et al. 2000).

2.2.5 Pneumococcal resistance to the immunological defence of the host

Once pneumococci have entered the human host, they need to protect themselves against the human immunological defence. The main role of the capsule is to protect pneumococci against phagocytosis (Wood et al. 1949). The pneumococcal capsular polysaccharides also inhibit activation of the alternative complement pathway (Hyams et al. 2010a). The essential role of the capsule in pneumococcal virulence is shown by non-encapsulated mutants, since the non-encapsulated mutants are more rapidly cleared from the circulation than encapsulated strains (Watson et al. 1990). In addition, non-encapsulated strains cause reduced densities of colonisation compared to encapsulated strains (Nelson et al. 2007b). The type of capsular polysaccharide, i.e. serotype, plays an important role in pneumococcal virulence. For instance, different serotypes vary in their resistance to the human complement-mediated defence (Melin et al. 2009; Hyams et al. 2010b; Melin et al. 2010a), explaining why a genetic switch of the capsular type changes the virulence properties of the bacterium (Kelly et al. 1994; Melin et al. 2010c). In addition, several pneumococcal proteins contribute to the inhibition of the deposition of complement on the pneumococcal surface (Tu et al. 1999; Jarva et al. 2003). PspC binds to factor H, a negative regulator of the alternative complement pathway (Janulczyk et al. 2000; Dave et al. 2001; Jarva et al. 2002; Lu et al. 2006), whereas the classical pathway may be down-regulated by PspA via inhibition of the deposition of the complement component C1q on the pneumococcal surface (Ren et al. 2003; Li et al. 2007). Further, Ply activates the classical complement pathway at distant sites from the bacterial surface, thereby depleting complement components and reducing the opsonic activity of serum (Paton et al. 1984; Mitchell et al. 1991; Yuste et al. 2005).

An additional mechanism of pneumococci to resist the human defence is the ability of PspA to bind lactoferrin, which protects pneumococci against the bactericidal effects of apolactoferrin (Hammerschmidt et al. 1999; Hakansson et al. 2001; Shaper et al. 2004). In addition, pneumococci are able to evade surveillance by human dendritic cells (DCs) on the mucosal surface by pneumolysin expression (Littmann et al. 2009).

2.3 Host defence against pneumococci

Pneumococci have many mechanisms by which they try to defeat the human immunological defence, but the human defence is also well equipped. Pneumococci enter the human host through the enormous area of mucosal surfaces of the

respiratory tract, which makes the first-line defence at the mucosal surfaces very important. If pneumococci invade tissue, an inflammatory response will be induced, which will destruct tissue and consume a lot of energy.

2.3.1 Mucosal immunity

The first-line defence against pneumococci occurs non-specifically on the mucosal surfaces via mucus, mucociliary transport, the flow of saliva, and via various soluble factors such as lactoferrin, lysozyme, cytokines and antibacterial peptides (e.g. β -defensins) (Canessa et al. 2010). The mucosal surfaces also contain a number of cells of the innate immune system, including, e.g. neutrophils, macrophages and DCs (Holmgren et al. 2005). Epithelial cells, DCs and macrophages have special receptors called toll-like receptors (TLRs) that recognise highly preserved molecular features called pathogen-associated molecular patterns (PAMPs) on pneumococci (Canessa et al. 2010). When the TLRs bind to PAMPs on pneumococci, a complex signalling pathway is induced and the immune response to the pathogen is activated (Calbo et al. 2010).

The specific adaptive immune system of the mucosa, called the mucosa-associated lymphoid tissue, lies below the mucosal surfaces. B-cells primed in the lymphoid tissue are seeded out through lymph and blood and home to the mucosal tissue and secretory glands, where they differentiate into immunoglobulin (Ig) producing plasma cells. The dominant Ig class in the mucosal immune system is IgA, which is produced almost exclusively as a dimer linked by a J-chain (Woof et al. 2006). The polymeric immunoglobulin receptors (pIgRs) transport the IgA dimers to the luminal side of the epithelium, where they are released along with a part of the receptor, a secretory component. The resulting secretory IgA (sIgA) molecule is more resistant to proteases than serum antibodies and is thus suitable for the harsh conditions on mucosal surfaces (Woof et al. 2006). sIgA prevents pneumococci and other pathogens from adhering to the host and neutralises bacterial toxins and enzymes.

Host response to the nasopharyngeal pneumococcal colonisation

The first step in the interaction between pneumococci and the human host is nasopharyngeal colonisation. The colonisation event may persist from days to months (Gray et al. 1980; Sleeman et al. 2006; Hogberg et al. 2007; Hill et al. 2008) and result in clearance of the colonising pneumococci or in pneumococcal disease. Pneumococcal carriage has been shown to act as an immunogenic stimulus resulting in production of serum and salivary antibodies to both capsular polysaccharide and

pneumococcal protein antigens in infants and young children (Rapola et al. 2000; Simell et al. 2001; Soininen et al. 2001; Simell et al. 2002; Adrian et al. 2004; Bogaert et al. 2006; Simell et al. 2006; Holmlund et al. 2007; Simell et al. 2009; Lebon et al. 2011). In addition, in adults, pneumococcal carriage has been shown to induce somewhat increased concentrations of serum anti-capsular and anti-protein IgG antibodies (McCool et al. 2003; Goldblatt et al. 2005). The role of these antibodies, however, is not entirely clear. It has been shown that antibodies are not required at all for the clearance of pneumococcal colonisation in mice (McCool et al. 2004; Malley et al. 2005), while in another study, both mucosal and systemic antibodies, as well as cellular host factors, have a role in protection against colonisation (Richards et al. 2010). In a study of experimental human carriage, it was shown that pre-existing serum anti-capsular IgG did not correlate with protection against nasopharyngeal carriage, but pre-existing levels of serum and mucosal antibodies to PspA were associated with protection against carriage (McCool et al. 2002). In Gambian and Israeli infants and children, at least some serotypes (14 and 23F) generate anti-capsular antibodies that might reduce the risk of carriage (Hill et al. 2008; Weinberger et al. 2008). In Bangladeshi infants and children, previous pneumococcal carriage was shown to protect against subsequent pneumococcal carriage, but in a serotype-independent manner (Granat et al. 2009).

Immunisations of infants with PCVs have been shown to reduce carriage of the pneumococcal vaccine serotypes (Dagan et al. 1996; Mbelle et al. 1999). Immunising B-cell depleted, but not T-cell depleted, mice with a pneumococcal whole-cell vaccine fully protected the mice against pneumococcal colonisation (Malley et al. 2005). This indicates that both antibodies and cellular host factors have a role in long-term protection against pneumococcal colonisation. In a mouse model, a killed pneumococcal whole-cell vaccine was shown to induce the CD4⁺ T_H17-cells to produce cytokine IL-17A (Lu et al. 2008). IL-17A induces the secretion of cytokines and chemokines by local epithelial cells, which attracts neutrophils to the site of infection (Yoichiro et al. 2008; Pappu et al. 2010). The effector cells clearing bacterial colonisation have been shown to be monocytes/macrophages (primary and secondary colonisation) and neutrophils (secondary colonisation) (Zhang et al. 2009). Thus, IL-17A may not prevent pneumococcal colonisation, but has been shown to reduce the density and duration of pneumococcal carriage (Lu et al. 2008).

2.3.2 Systemic immunity

If pneumococci succeed in penetrating the mucosal surface, the human host has many mechanisms to defeat the intruder. These mechanisms can be divided into

antibody-independent and antibody-dependent defence. The antibody independent defence is fast but not very specific, whereas the antibody-dependent defence is specific but slow.

Antibody-independent immunity against pneumococci

Pneumococcal pneumonia is characterised by an intense inflammatory response of the lungs induced by pneumococcal components, mainly the pneumococcal cell wall. The pneumococcal components are recognised by alveolar cells, which start to produce cytokines. Cytokines are important small proteins that regulate the immune responses. TNF- α and IL-1, produced by the alveolar macrophages, induce chemokines (e.g. IL-8) which attract neutrophils to the site of infection. In established pneumonia, neutrophils become the major cells phagocytosing bacteria (Calbo et al. 2010). Macrophages also secrete IL-6, which participates in the anti-pneumococcal defence of the lung by inducing acute phase proteins, including C-reactive protein (CRP) (Calbo et al. 2010). CRPs are produced by the liver, circulate in the blood, and play an important role in the defence against invasive pneumococcal infection by binding to the cell wall of pneumococci, thereby opsonising them. The CRPs then activate the classical complement pathway and promote opsonophagocytosis of the pneumococci by neutrophils (Volanakis et al. 1974; Krarup et al. 2005). DCs are important in initiating the adaptive humoral responses to both pneumococcal protein and polysaccharide antibodies (Colino et al. 2002).

The complement system has a role in both innate and adaptive immunity. Individuals deficient in complement components have an increased susceptibility to recurrent pneumococcal infections (Janoff et al. 1997). The classical pathway is the dominant route of complement activation in the human defence against pneumococci (Brown et al. 2002). Antibodies bound to pneumococci activate the first complement component of the classical pathway, C1q, which activates the complement cascade leading to C3b deposition on the pneumococcal surface. C1q may also bind to CRP which has bound to PCCho of the pneumococcal cell wall, and may thus activate the complement cascade even without antibodies (Volanakis et al. 1974; Mold et al. 2002). The complement cascade can also be activated directly by microbes via the alternative complement pathway, leading to clustering of C3b molecules on the pneumococcal surface (Winkelstein et al. 1980).

The C3 degradation product C3a recruits inflammatory cells to the site of infection, whereas the degradation products C3b, iC3b and C3d opsonise pneumococci (Fearon et al. 1983; Toapanta et al. 2006). Phagocytic cells then bind to opsonised pneumococci via complement receptors (CRs) and engulf the bacteria.

B-cells bear CR2 for binding C3d-opsonised bacteria (Griffioen et al. 1991). When pneumococcal polysaccharides bind to the Ig molecule on the surface of the B-cell, the C3d-CR2 binding functions as a co-activation signal (Knopf et al. 2008) in the activation of antibody-producing B-cells (Griffioen et al. 1991; Mitsuyoshi et al. 2005). Young children express CR2 poorly, which may in part explain their poor response to the polysaccharide antigens (Griffioen et al. 1992; Griffioen et al. 1993).

Pneumococcal clearance from the lungs mainly results from type-specific antibody- and complement-mediated phagocytosis of the pneumococci by neutrophils and alveolar macrophages (Bogaert et al. 2004). Opsonised pneumococci in the blood are transferred by erythrocytes to the spleen or liver for opsonophagocytosis by the resident macrophages (Hament et al. 2003; Li et al. 2007). The absence of the spleen or cirrhosis of the liver increases the risk of severe pneumococcal infections (Bruyn et al. 1992).

Antibody-dependent immunity against pneumococci

Antibodies are produced by B-cells. Protein antigens require T-cell help for B-cell activation and are thus called T-cell dependent (TD) antigens. Briefly, when the B-cell receptor on the surface of a B-cell binds to a pneumococcal antigen, the receptor and the antigen are internalised and processed. Peptides from the pneumococcal antigen are then expressed on the surface-located major histocompatibility complex class II (MHCII) molecules of the B-cell. A CD4⁺ T-helper cell that has been primed by the same antigen binds to the B-cell receptor with its T-cell receptor. This induces the T-cell to secrete cytokines that cause the B-cell to somatically mutate in response to the antigen and proliferate into pneumococcal protein-antibody secreting plasma cells or memory cells. Upon re-encounter with pneumococci or after re-immunisation with a PCV, the memory B-cells respond quickly and differentiate into high affinity antibody producing plasma cells (Elgueta et al. 2010). The immunity induced by TD antigens is long lasting; memory B- and T-cells are generated and antibodies of multiple isotypes with high affinity are produced (Lesinski et al. 2001). Adults as well as children of all ages are able to respond to the TD protein antigens (Rapola et al. 2000; McCool et al. 2003; Baril et al. 2004b).

B-cells can also be activated by antigens for antibody production without T-cell help (Stein 1992). The T-cell independent (TI) antigens can be divided into type 1 (TI-1) and type 2 (TI-2). TI-1 antigens are, e.g. lipopolysaccharides, which activate a non-specific B-cell response via the TLR (Lesinski et al. 2001). TI-2 antigens are, e.g. pneumococcal polysaccharides. Polysaccharides fail to associate with the MHCII molecules and thus do they not activate the T-cells. Polysaccharide antigens contain multiple identical epitopes that are able to cross-link the surface-exposed Igs on polysaccharide-specific mature B-cells, and thereby activate B-cells to produce

antigen-specific antibodies (Lesinski et al. 2001). The TI-2 antigens induce only low levels of somatic hypermutations. It was thought for a long time that memory B-cells are not generated during TI-2 immune responses, but a few years ago memory B-cells actually were shown to be generated (Obukhanych et al. 2006). The development of memory B-cells is T-cell independent and the generated memory B-cells are phenotypically distinct from those elicited by protein antigens. The distinct characteristic of these TI-2-induced memory B-cells is increased quantity, rather than quality, of antigen-specific clones. Since polysaccharides are retained in the body for long periods of time, memory B-cells capable of responding to such persistent antigens necessitate a suppressive mechanism because continuous reactivation would lead to antibody overproduction. Therefore, these memory B-cells are strictly suppressed by antigen-specific IgG antibodies (Obukhanych et al. 2006).

B-cells that respond to TD antigens are called conventional B-2 cells, while B-cells that respond to TI-2 antigens are called B-1 cells and marginal zone B-cells (Fagarasan et al. 2000; Haas et al. 2005). Marginal zone B-cells are found in adults, but are rare in infants, thus limiting the responses of infants to the TI-2 antigens (Siegrist 2007).

Pneumococcal antibodies of different isotypes are produced. IgM is a pentamer that effectively activates complement and is important in early infection. IgG is the most prevalent isotype found in four subclasses: IgG1–4, subclass IgG1 being the most prevalent (Plebani et al. 1989). There are differences in complement fixation as well as in IgG-receptor (FcγR) binding between the four IgG subclasses. IgG1 and IgG3 are generally induced in response to protein antigens, whereas IgG2 and IgG4 are associated with polysaccharide antigens (Schroeder et al. 2010). IgA functions mainly as a neutralising antibody and is found in two subclasses: IgA1 and IgA2.

The IgG molecule is composed of two longer heavy and two shorter light chains (Fig. 4). The N-terminal parts of the heavy chain together with the light chains are called the antigen-binding (Fab) fragments, whereas the C-terminal ends of the heavy chains are called the crystallisable (Fc) non-antigen binding fragment. The two heavy chains are joined by two disulphide bonds at the flexible hinge region, allowing independent movement of the two Fab fragments (Murphy et al. 2008). The N-terminal ends of the Fab fragments are the regions of antigen-binding that vary extensively and are therefore known as the variable region. The rest of the antibody molecule does not vary as much, and is thus called the constant region. The variable domains are created by complex gene rearrangements and are subjected to somatic hypermutations after exposure to antigens to allow affinity maturation (Neuberger 2008; Schroeder et al. 2010).

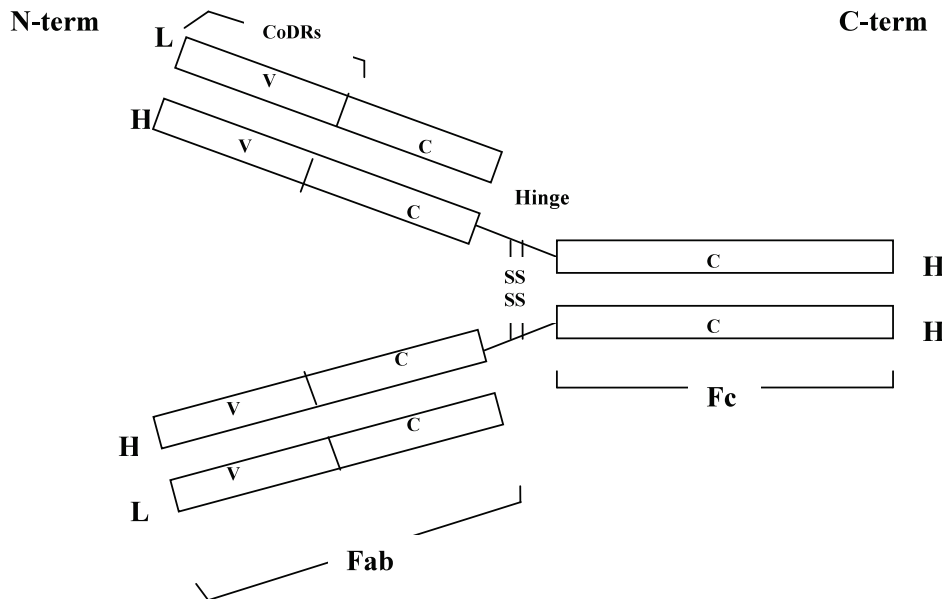


FIGURE 4. Structure of an IgG molecule. L = light chain, H = heavy chain, V = variable region, C = constant region. CoDRs = complementarity-determining regions. The two heavy chains are connected with disulphide bonds. N-term = amino-terminal end, C-term = carboxy-terminal end. Modified from (Schroeder et al. 2010).

Each variable domain has three regions of high sequence variability known as the complementarity-determining regions (CoDRs). The three CoDRs of the heavy chain are paired with the three CoDRs of the light chain to form the antigen-binding site. The constant region of the antibody specifies the effector function, such as complement activation and FcγR binding. The constant domains may be switched to alter the effector function while maintaining the antigen specificity. The heavy chain constant region also defines the isotype of the Ig molecule (Schroeder et al. 2010).

2.4 Prevention of pneumococcal infections

The development of pneumococcal vaccines had already begun at the beginning of the 20th century. The discovery of penicillin and other antibiotics stopped the vaccine development for a few decades, since pneumococcal diseases could successfully be treated with antibiotics. However, in the 1960s, the first non-susceptible pneumococcal strains were detected, after which the resistance to antibiotics spread rapidly worldwide. This raised the interest in pneumococcal

vaccines again and in 1977, the first pneumococcal polysaccharide vaccine was licensed.

Today, pneumococcal infections are still treated with antibiotics, but the several multiresistant pneumococcal strains sometimes make treatment difficult. However, the pneumococcal resistance to penicillin and other antibiotics varies between countries: in the Philippines, the penicillin resistance is only 1%, while in the Republic of Korea, more than 50% of the pneumococcal strains are resistant to penicillin (Bravo 2009). The degree of resistance also varies between serotypes: The highest rates of resistance to penicillin and erythromycin worldwide have been found in serotypes 6B, 6A, 9V, 14, 15A, 19F, 19A and 23F (Liñares et al. 2010). Since pneumococci are able to take up external DNA and incorporate it into their own genomes (Claverys et al. 2002), the resistance genes can be transferred from one strain to another. To prevent the spread of the multiresistant serotypes, prevention of pneumococcal infections is important. This prevention can be carried out through vaccinations.

2.4.1 Systemic immunisation

Active or passive immunisation may be used in the prevention of pneumococcal diseases. Of these, only active immunisation gives a long-lasting immunity. At its best, immunisation against pneumococci evokes both the systemic and mucosal arms of the immune system. When an immunised person acquires pneumococci, in most cases his/her immune response rapidly clears the bacteria and thereby prevents the disease.

Pneumococcal polysaccharide vaccines

The first licensed pneumococcal vaccine was a 14-valent pneumococcal polysaccharide vaccine. Today, PPV is the only polysaccharide vaccine in use. Although polysaccharides are TI-2 antigens, they confer protection against IPD in human adults (Melegaro et al. 2004a). However, secondary immunisation does not elicit a boost in antibody production (O'Brien et al. 1996). PPV also fails to reduce nasopharyngeal carriage of pneumococci (Herva et al. 1980; Wright et al. 1981). In children less than two years of age, PPV is poorly protective due to low immunogenicity of polysaccharides in this immunologically immature age group (Sell et al. 1981; Douglas et al. 1983; Koskela et al. 1986; Temple et al. 1991). However, in a study conducted in Papua New Guinea, the 14- and 23-valent pneumococcal polysaccharide vaccines were able to reduce mortality due to acute

lower respiratory tract infections by 59% and 50% in children aged less than five years and two years, respectively (Riley et al. 1986).

PPV is currently recommended in at least the US and Finland for all persons aged over 65 years, and for persons aged 2–64 years with underlying medical conditions after receiving the recommended doses of the PCV in infancy (CDC et al. 2010; Nohynek et al. 2010; Nuorti et al. 2010).

Pneumococcal conjugate vaccines

Conjugation of the polysaccharide antigen to a protein carrier changes the nature of the antigen from TI to TD. Currently, there are three licensed PCVs on the market: 7-, 10- and 13-valent PCVs (PCV7, PCV10 and PCV13). PCV7 contains serotypes 4, 6B, 9V, 14, 18C, 19F and 23F. It has been very successful in reducing the incidence of invasive disease and AOM caused by the vaccine serotypes in children aged less than two years (Black et al. 2001; Eskola et al. 2001; Whitney et al. 2003). PCV7 has been shown to be immunogenic even in newborns (Pomat et al. 2010; Warira et al. 2010), although it is not recommended for infants before the age of six weeks. Due to herd immunity, the rates of vaccine serotype IPD cases have also decreased in unimmunised adults (Lexau et al. 2005). Additionally, PCV7 has reduced the use of antibiotics due to a decline in disease rates and thus has also the rates of antibiotic-resistant pneumococci declined (Liñares et al. 2010). PCV10 contains the same serotypes as PCV7 complemented with serotypes 1, 5 and 7F, and PCV13 is further complemented with serotypes 3, 6A and 19A. Both PCV10 and PCV13 are safe and immunogenic in infants and their additional serotypes increase the overall serotype coverage as compared to PCV7 (Prymula et al. 2009; FDA 2010; Reinert et al. 2010; Yeh et al. 2010; Lagos et al. 2011).

PCVs are now widely in use, but even they are not the most optimal vaccines. In high-income countries, PCV7 has been a success, but the variation in serotype distribution in different parts of the world leads to a low coverage against IPD in some regions (Johnson et al. 2010). Thus, PCVs with wider coverage are needed and PCV10 and PCV13 try to solve this problem. Further, the serotypes common in one age group are not necessarily common in others. In a German study, PCV7 covered about 70% of the serotypes in the age group 6–24 months, 50–60% in the age group 3–5 months and only 20–40% in the age group 0–2 months (Von Kries et al. 2002). Finally, large-scale immunisations with PCV7 have led to serotype replacement, i.e. an increase in the incidence rates of IPD cases caused by non-vaccine serotypes, including the multiresistant serotype 19A, which is now included in PCV13 (Moore et al. 2008; Kaplan et al. 2010; Liñares et al. 2010). PCVs have been shown to reduce pneumococcal carriage (Dagan et al. 1996; Mbelle et al. 1999), but the vaccine serotype carriage is now being replaced by non-vaccine serotypes (Nahm et

al. 2009; WHO 2010). Adding serotypes to PCVs may cause previously unusual serotypes to become common and totally new serotypes to emerge due to selective pressure. Concordantly, AOM caused by bacteria other than pneumococci, e.g. *Staphylococcus aureus*, have increased after the large-scale immunisations with the PCVs began (Veenhoven et al. 2003).

The PCVs may not be the optimal choice for low-income countries, since these vaccines are currently complicated and expensive to manufacture and they require refrigeration as well as multiple injections. To reduce the high costs of the PCVs, immunisations with fewer than the recommended three or four doses (i.e. alternative schedules) have been evaluated (Russell et al. 2009; Deceuninck et al. 2010; Givon-Lavi et al. 2010; Ota et al. 2011). In the Philippines, a one-dose schedule versus a three-dose schedule of an investigational PCV11 was evaluated: at nine months of age; the antibody concentrations induced by one PCV11 dose given at 18 weeks of age were similar to antibody concentrations induced by three PCV11 doses given at the ages of 6, 10 and 14 weeks (Lucero et al. 2004).

Pneumococcal protein vaccine candidates

The limitations of the polysaccharide-based vaccines have led to the search for alternative pneumococcal vaccines that would be both cost effective and potentially protect infants and children against all pneumococcal serotypes. A vaccine with one or more of the conserved pneumococcal protein antigens might fulfil these criteria and have the potential to provide protection against nasopharyngeal carriage and IPD with global serotype coverage (Crain et al. 1990; Morrison et al. 2000). A protein-based vaccine might be easier and cheaper to manufacture as compared to the PCVs. Moreover, proteins are TD antigens and thus, in contrast to the polysaccharide vaccines, also have the potential to offer protection in infants and young children. Currently, several pneumococcal proteins are being studied as potential vaccine candidates, either as such or in combination with other pneumococcal proteins (Briles et al. 2003; Ogunniyi et al. 2007b; Godfroid et al. 2011; Gong et al. 2011). Six of the potential pneumococcal vaccine candidate proteins are included in the present work (PspA, PsaA, Ply, PspC, PhtD and LytC) and they will be described in detail below.

Pneumococcal surface protein A (PspA)

PspA is a highly variable surface protein which is found on all clinical isolates of pneumococci studied to date (Crain et al. 1990). The molecular size of PspA varies from 67 to 99 kDa in different pneumococcal strains (Waltman et al. 1990). PspA has five distinct regions: an amino-terminal signal peptide directing the protein to the outer surface, an alpha-helical functional domain, a proline-rich region believed to span the cell wall, a choline-binding domain composed of multiple highly

conserved amino acid repeats (usually 10 repeat regions of approximately 20 amino acids) anchoring the protein to the cell surface and finally a carboxy-terminal hydrophobic tail (Yother et al. 1992) (Fig. 5).

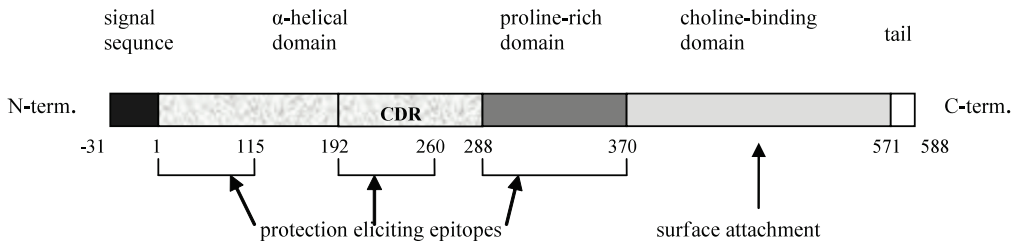


FIGURE 5. Amino acid sequence of PspA of strain Rx1. CDR = clade defining region. Figure based on (Yother et al. 1992), (Hollingshead et al. 2000) and (Daniels et al. 2010a).

The amino-terminal functional domain exhibits more variability than the other parts of the protein. Based on the diversity of the PspA molecules, they have been divided into three families and further into six clades (Hollingshead et al. 2000). The six clades diverge from each other by more than 20% at the clade defining region (CDR) (Hollingshead et al. 2000) (Fig. 5). Approximately 95% of all PspA proteins are grouped into families 1 and 2 (Vela Coral et al. 2001; Brandileone et al. 2004; Mollerach et al. 2004; Melin et al. 2008b).

The amino-terminal functional end protrudes outside the capsule (Jedrzejewski et al. 2000; Gor et al. 2005), and thus the capsule does not block the binding of antibodies specific to PspA (Daniels et al. 2006) (Fig. 2). The protection-eliciting epitopes of PspA have been localised to the first 115 amino acids of the alpha-helical region and to the clade-defining region (McDaniel et al. 1994; Roche et al. 2003a; He et al. 2007) (Fig. 5). Recently, the proline-rich region has also been shown to bear protection eliciting epitopes (Daniels et al. 2010a) (Fig. 5).

PspA is highly immunogenic in mice (Briles et al. 1996; Tart et al. 1996; Gor et al. 2005). The intranasal immunisation of mice with PspA induces mucosal and systemic immune responses that protect against nasopharyngeal carriage, as well as against systemic disease (Wu et al. 1997). Immunisation of rats with PspA has been shown to induce protection against pneumococcal AOM (White et al. 1999).

Natural anti-PspA antibodies have been shown to exist in the sera of human adults and children (Virolainen et al. 2000). In human adults, PspA has been shown to be immunogenic after intramuscular immunisation with recombinant PspA (Nabors et al. 2000), as well as after experimental human carriage with pneumococci (McCool

et al. 2002). Serum from human adults immunised with PspA was shown to protect mice against pneumococcal infection (Briles et al. 2000c). In addition, naturally acquired human salivary anti-PspA antibodies may have a role in protection against pneumococcal AOM in children (Simell et al. 2007).

Pneumococcal surface adhesin A (PsaA)

PsaA is a highly conserved small (37-kDa) lipoprotein (Sampson et al. 1997) which is expressed by all serotypes of pneumococci (Sampson et al. 1997; Morrison et al. 2000). PsaA is part of an ABC-type manganese permease complex, which imports manganese into pneumococcal cells (Dintilhac et al. 1997; Lawrence et al. 1998; McAllister et al. 2004). Manganese has a central role in the regulation of the stress responses, physiology and metabolism of pneumococci (Johnston et al. 2004; Ogunniyi et al. 2010).

PsaA protrudes from the cell membrane (Lawrence et al. 1998), but it may be buried underneath the thick cell wall and capsule (Jedrzejewski 2001). Gor and co-workers could not detect PsaA on the pneumococcal surface using flow cytometry (Gor et al. 2005). Despite this, it has been found that monoclonal anti-PsaA antibodies bind to pneumococci (Russell et al. 1990), and the anti-PsaA antibodies are able to inhibit binding of pneumococci to human nasopharyngeal cells (Romero-Steiner et al. 2003; Romero-Steiner et al. 2006). In addition, E-cadherin, which is present on human nasopharyngeal cells, has been shown to bind to PsaA (Anderton et al. 2007). Recombinant E-cadherin was demonstrated to bind to and block pneumococci from adhering to nasopharyngeal cells (Anderton et al. 2007). This indicates that PsaA is surface exposed.

PsaA has been shown to be immunogenic in mice after immunisations (Gor et al. 2002) and after nasopharyngeal carriage (Palaniappan et al. 2005). Immunisation of mice with PsaA alone or in combination with other pneumococcal virulence proteins has been shown to protect mice against death (Talkington et al. 1996; Ogunniyi et al. 2000); on the other hand, some other studies have been contradictory (Briles et al. 2000b). Several studies have shown that immunisation of mice with PsaA or peptides of PsaA induce protection against pneumococcal carriage (Briles et al. 2000a; Briles et al. 2000b; De et al. 2000; Seo et al. 2002; Pimenta et al. 2006). In addition, intranasal immunisation of mice with chitosan DNA nanoparticles expressing PsaA was shown to inhibit pneumococcal carriage (Johnson et al. 2002; Xu et al. 2010). Recently, mice immunised with PsaA and PCV7 showed reduced pneumococcal carriage of non-vaccine serotype 19A (Whaley et al. 2010). The protection offered by PsaA immunisations has been suggested to vary between strains (Gor et al. 2005).

PsaA has also been shown to be immunogenic in humans. Children aged 6–24 months of age generated serum IgG antibodies to PsaA in response to pneumococcal carriage and AOM (Rapola et al. 2000). In addition, secretory IgA antibodies to PsaA have been found in the saliva of children after culture-confirmed pneumococcal exposure (carriage or AOM) and in the saliva of adults (Simell et al. 2001). Furthermore, anti-PsaA antibodies have been shown to be associated with decreased risk of pneumococcal carriage progression to pneumococcal AOM in children >9 months old (Rapola et al. 2001a).

Pneumolysin (Ply)

Ply is a 53 kDa pore-forming toxin produced by most clinical strains of pneumococcus (Kancierski et al. 1987; Walker et al. 1987). Earlier, Ply was thought to be highly conserved, but today at least 15 different alleles of Ply have been isolated (Jefferies et al. 2007). Different alleles have been found in strains associated with carriage than in strains associated with invasion of the host (Jefferies et al. 2010). The degree of haemolytic activity varies between the Ply alleles, and alleles for totally non-haemolytic Ply have been found (Jefferies et al. 2007).

Since Ply is a toxin, it cannot be used as a vaccine antigen as such. A nontoxic variant of Ply (a pneumolysoid) has the potential to induce neutralising antibodies and to function as an adjuvant. Therefore, different mutations that reduce the cytotoxicity of Ply have been evaluated. The W433F mutation reduces the haemolytic activity to 0.1–1%, as compared to the native Ply (Paton et al. 1991; Mitchell et al. 1992). This mutant, called PdB, is immunogenic and provides protection against pneumococcal challenge in animal models (Alexander et al. 1994; Ogunniyi et al. 2000; Ogunniyi et al. 2001). Another mutant, PdT, containing two additional mutations besides the W433F (D358N and C428G), showed 0.0001% cytotoxicity compared to the wild-type Ply (Berry et al. 1995). PdT fused to PsaA and conjugated to CPS was shown to protect mice against pneumococcal colonisation and fatal pneumonia (Lu et al. 2009).

A genetically detoxified Ply mutant was reported by Michon and co-workers, but the exact mutation has not been identified (Michon et al. 1998). Kirkham and co-workers reported another nontoxic Ply mutant, in which amino acid A146 was deleted, resulting in the blockage of pore formation (Kirkham et al. 2006). This pneumolysoid was shown to be immunogenic and protective in a mouse model, which makes it a promising vaccine candidate (Kirkham et al. 2006; Wu et al. 2010). For clarity, in this thesis, I will use the short form ‘Ply’ for all different forms of pneumolysin and pneumolysoids.

In humans, Ply has not been used for immunisation, but purified naturally acquired human anti-Ply IgG antibodies have been shown to protect mice against

pneumococcal infection (Musher et al. 2001). Low CD4⁺ T-cell immunity to Ply in peripheral blood mononuclear cells from children has been shown to be associated with pneumococcal nasopharyngeal carriage (Zhang et al. 2007). In adults, the same phenomenon could not be shown (Mureithi et al. 2009).

Pneumococcal surface protein C (PspC)

PspC is a highly polymorphic protein that has strong molecular and serologic similarities with PspA (Brooks-Walter et al. 1999). Sequence analyses have shown that there are many allelic variants of PspC, and they have been grouped into 11 major subgroups (Iannelli et al. 2002). The *pspC* gene is present in 75–100% of all pneumococcal strains (Brooks-Walter et al. 1999; Iannelli et al. 2002). Several properties have been attributed to PspC under different names: pneumococcal surface protein C (PspC), choline-binding protein A (CbpA), *S. pneumoniae* secretory IgA binding protein (SpsA), C3-binding protein A (PbcA) and factor H-binding inhibitor of complement (Hic) (Hammerschmidt et al. 1997; Rosenow et al. 1997; Brooks-Walter et al. 1999; Cheng et al. 2000; Janulczyk et al. 2000). Since PspC is a generic name referring only to the surface location of the protein, I will use the name PspC throughout this thesis.

The structure of PspC can be divided into four distinct regions: an amino-terminal 37 amino-acid signal peptide, an alpha-helical region, a proline-rich region and a carboxy-terminal anchor responsible for the attachment to the cell surface. The mechanism for attachment is not uniform among the different PspC proteins, since some proteins bear a choline binding anchor and some an LPXTG anchor (Iannelli et al. 2002).

In a mouse model, PspC is able to elicit protection against nasopharyngeal colonisation and death when challenged with the highly virulent pneumococcal strain D39 (Ogunniyi et al. 2001; Balachandran et al. 2002). In addition, mice challenged with a PspC-negative mutant showed significantly increased survival rates as compared to mice challenged with the wild-type strain (Iannelli et al. 2004). While many studies have focused on the alpha-helical parts of the PspC proteins, a recent study showed that the carboxy-terminal proline-rich regions are also able to elicit antibody-mediated protection against sepsis (Daniels et al. 2010a).

In a model of experimental human carriage, an antibody response to PspC was generated in adults, indicating that the protein is exposed and immunogenic (McCool et al. 2003). Culturing adenoidal lymphocytes from 20 children in a concentrated pneumococcal culture supernatant containing pneumococcal proteins stimulated the production of anti-PspC antibodies, suggesting that PspC may be a good upper respiratory mucosal antigen in children (Zhang et al. 2002). In children

aged 12–18 months, a higher concentration of PspC has been suggested to protect against subsequent pneumococcal AOM (Simell et al. 2009).

Pneumococcal histidine triad protein D (PhtD)

PhtD belongs to the family of highly conserved surface-exposed pneumococcal proteins that have a histidine-triad motif in their amino acid sequences (Adamou et al. 2001). The Pht-protein family consists of proteins PhtA, PhtB, PhtD and PhtE. In the literature, different names for the members of this protein family have been used: PhtA has also been designated as Sp36 and BVH-11-3, PhtB as PhpA and BVH-11, PhtD as BVH-11-2 and PhtE as BVH-3 (Adamou et al. 2001; Wizemann et al. 2001; Zhang et al. 2001; Hamel et al. 2004). The biological functions of these proteins are unclear, but they have been suggested to be involved in invasion and pathogenesis (Hava et al. 2002; Panina et al. 2003). More specifically, they have been suggested to be involved in the inhibition of complement deposition by binding to factor H (Ogunniyi et al. 2009). However, Melin et al. could not confirm that these proteins would have a major role in the inhibition of complement or binding of factor H; their contribution depended on the bacterial genotype (Melin et al. 2010b).

In a mouse model, PhtD elicits protection against pneumococcal systemic infection caused by serotypes 3, 4, 6A and 6B (Adamou et al. 2001; Ogunniyi et al. 2009). In addition, immunisation with PhtD has been shown to result in higher survival rates after lethal intranasal challenge (Denoël et al. 2011). A significant additive effect on protection was observed with the combination of PhtD vaccination and the injection of anti-polysaccharide antibodies specific for serotypes 1 and 3 (Denoël et al. 2011). In a mouse nasopharyngeal and lung colonisation model, the PhtD protein was found superior to PspC and PspA in terms of protection and serotype coverage (Godfroid et al. 2011).

In humans, anti-PhtD antibodies have been detected in the convalescent-phase sera of three out of five infants and children with pneumococcal bacteraemia, indicating that this protein is exposed and recognised by the immune system during pneumococcal disease (Adamou et al. 2001). In addition, pneumococcal carriage and AOM have been shown to induce anti-PhtD serum antibodies (Simell et al. 2009). Moreover, a fragment of the PhtD protein reacted with the anti-PhtD antibodies in 83% of 30 serum samples from healthy adults (Beghetto et al. 2006). Additionally, human naturally acquired anti-PhtD antibodies transferred into mice demonstrated a significant protection against lethal intranasal challenge (Godfroid et al. 2011). Pneumococcal carriage and AOM have also been shown to induce serum antibodies to two other pneumococcal histidine triad proteins, PhtB and PhtE (Holmlund et al. 2007).

Lysozyme (LytC)

LytC is a lysozyme that belongs to the family of choline-binding proteins (Gosink et al. 2000). It is an autolytic enzyme that degrades the cell wall of pneumococci (Garcia et al. 1999) and participates in the lysis of target cells (Eldholm et al. 2009). Pneumococcal strains mutated for *lytC* and *pspC* have been shown to be poor biofilm formers (Moscoso et al. 2009). LytC comprises an amino-terminal signal peptide, a cell wall-binding domain and a carboxy-terminal catalytic domain (Monterroso et al. 2005; Monterroso et al. 2008). To become a stable enzyme, the cell wall-binding domain needs to bind to the CPho of the cell wall lipoteichoic and teichoic acids (Monterroso et al. 2008).

In a rat model, LytC has been shown to promote pneumococcal colonisation of the nasopharynx (Gosink et al. 2000). Mice immunised with LytC were shown to be protected against a lethal challenge with serotype 6B pneumococci (Wizemann et al. 2001). In adherence experiments with human nasopharyngeal Detroit cells, a LytC deletion mutant showed 70% loss of adherence (Gosink et al. 2000). The detection of antibodies in the majority of the 17 convalescent-phase serum samples from patients recovering from pneumococcal pneumonia suggests that LytC is expressed *in vivo* and is immunogenic during disease in humans. Additionally, LytC has also been shown to be serologically cross-reactive among pneumococcal strains of different capsular serotypes (Wizemann et al. 2001).

Combinations of protein vaccine candidates

Immunisation with a combination of different pneumococcal protein antigens would probably induce the most efficient protection against pneumococci. In several immunisation studies, a synergistic or additive effect has been shown by using multiple proteins, even if the individual proteins had shown only little value by themselves. Immunisation of mice with a combination of PspA and Ply induced better protection against pneumococcal pneumonia as compared to the immunisations with these proteins alone (Briles et al. 2003). Immunisations with a combination of Ply, PspA and PspC showed significantly better protection against IPD when compared to the immunisations with only one or two of these proteins (Ogunniyi et al. 2007b; Ogunniyi et al. 2007a). In addition, mice immunised with a combination of PspA, PspC and the caseinolytic protease ClpP survived significantly longer than mice immunised with only one or two antigens (Cao et al. 2007).

2.4.2 Mucosal immunisation

The first-line defence against pneumococci occurs at the mucosal surfaces. Therefore, stimulation of mucosal immunity is a desirable goal of vaccination. A challenge of mucosal immunisation is that the antigen is rapidly destructed or inactivated by mucosal enzymes, which renders the immune responses insufficient. In order to enhance the immune responses, various mucosal adjuvants have been explored. Oral immunisation of mice with PspA or PsaA with cholera toxin as an adjuvant induced protective immunity against pneumococcal infection (Yamamoto et al. 1997; Seo et al. 2002). In addition, intranasal immunisation of mice with PspA or PsaA and the B subunit of cholera toxin has been shown to protect against pneumococcal colonisation (Wu et al. 1997; Pimenta et al. 2006). Intranasal immunisation of mice with a mixture of PspA and a TLR agonist induced PspA-specific antibodies and enhanced the clearance of bacteria from the airways (Oma et al. 2009). The response to intranasal immunisation with PspA has also been characterised as increased secretion of IL-17 and IFN-gamma by lung and spleen cells, respectively (Ferreira et al. 2009). The mucosal immunisation of mice with *Salmonella*-derived outer membrane vesicles containing PspA was shown to protect against pneumococcal challenge (Muralinath et al. 2011). Ply has also been evaluated as an adjuvant. A nontoxic form of Ply could act as a mucosal adjuvant to increase the local and systemic humoral response to genetically fused protein antigens (Douce et al. 2010).

2.4.3 Maternal and neonatal immunisations to protect the very young

To decrease childhood mortality and morbidity, protection of very young individuals against infection is of the utmost importance. One solution to circumventing the obstacles in protecting the very young could be maternal immunisation.

Maternal immunisation

Immunisation of the mother during pregnancy results in the transfer of elevated antibody concentrations through the placenta to the foetus (Saji et al. 1999), which may confer protection to the newborn. After birth, the infant will also receive antibodies from the mother through breast milk (Finn et al. 2002). Furthermore, by protecting the mother from acquiring infections, the infants will also be better protected.

The rates of neonatal and early infant deaths are especially high in low-income countries and a substantial proportion of these deaths are due to infections (Bahl et

al. 2005). In low-income countries, the prevalence of at least some breastfeeding of infants <6 months old is about 94% (Lauer et al. 2004). For these reasons, the role of maternal immunisation may be particularly important in low-income countries. In addition, infants and children in resource-poor settings often receive their own vaccinations late and are not necessarily given all the scheduled doses (Clark et al. 2009), which further highlights the need of early protection, for instance via maternal antibodies. However, maternal immunisation is not free of constraints. Malaria has been shown to suppress the immune responses to various antigens, including polysaccharides (Greenwood 2003). In some regions, the rates of HIV infection are high among pregnant women and HIV infection may impair the immune response of the mother, resulting in reduced antibody concentrations in the foetus (Greenwood 2003). In addition, premature births tend to be more common in resource-poor countries, while the active transfer of maternal antibodies to the foetus primarily occurs during the last few weeks of pregnancy (Greenwood 2003). There has also been discussion about whether or not the maternal immunisation poses a risk to the developing foetus. The “Guidelines for vaccinating pregnant women” by the Advisory Committee on Immunisation Practices (ACIP) state that the risk of maternal immunisation is primarily theoretical and that the benefits of vaccination usually outweigh the potential risks in circumstances where the likelihood of disease exposure is high (CDC 2007).

Globally, maternal immunisation has proved to be highly successful in prevention of neonatal tetanus. WHO recommends that all pregnant women in low-income countries should receive at least two doses of tetanus vaccine, and during subsequent pregnancies, a complete course of five doses (Mulholland 1998). In addition, the influenza vaccine has been demonstrated to be safe and effective in pregnant women (Tamma et al. 2010), and ACIP recommends that it should indeed be given to all pregnant women (CDC 2007). Since the influenza vaccine is not licensed for infants less than six months of age, antibodies transferred from the mother to the infant play an essential role in protection of the child. A Bangladeshi study showed that immunisation of the mother with an inactivated influenza vaccine reduced the diagnosed influenza illnesses by 63% in infants up to six months of age (Zaman et al. 2008). PPV has been shown to be safe in pregnant women (Shahid et al. 1995; O'Dempsey et al. 1996; Munoz et al. 2001; Lehmann et al. 2002), and the ACIP recommends that it should be used for the immunisation of pregnant women belonging to the risk group for pneumococcal disease (CDC 2007; Nohynek et al. 2010).

Transfer of maternal antibodies to the infant

Maternal IgG antibodies (predominantly IgG1) passively diffuse and are actively transported across the placenta directly into the bloodstream of the foetus (Kohler et

al. 1966). The transfer of maternal antibodies provides the newborn with passive immunity against various infectious pathogens. The selective transport of IgG from the mother to the foetus is based on the function of a specific IgG transport protein in the placenta, FcRn (Roberts et al. 1990; Baker et al. 2009). This protein binds to the Fc portion of the IgG molecule. The IgG molecule is endocytosed and the pH change releases IgG into the umbilical cord (Baker et al. 2009). IgG transport from the mother to the foetus starts at approximately week 16 of gestation and increases thereafter (Saji et al. 1999). Most of the antibodies are acquired from the mother during the third trimester of pregnancy and the bulk of IgG transport to the foetus occurs during the last four weeks of pregnancy (Malek et al. 1994; Saji et al. 1999). At birth, the foetal IgG1 concentration may exceed that of the mother, while the IgG2 concentration is often lower than that of the mother (Saji et al. 1999; Simister 2003).

Neonatal immunisation

Currently, only a few vaccines are licensed for administration to human neonates. The Bacille Calmette-Guérin (BCG) vaccine against tuberculosis, the oral polio vaccine (OPV) and the hepatitis B vaccine (HBV) are given in some countries in the neonatal period (WHO 2011). In addition, administration of PCV7 to newborn Papua New Guinean and Kenyan babies was shown to be safe (van den Biggelaar et al. 2009; Warira et al. 2010). In a neonatal mouse model, intranasal administration of a pneumococcal serotype 1 conjugate vaccine along with a nontoxic enterotoxin adjuvant protected the neonate mice against pneumococcal infection (Jakobsen et al. 2002). In another neonatal mouse model, immunisation with a killed pneumococcal whole-cell vaccine reduced pneumococcal colonisation in the neonate mice (Bogaert et al. 2009).

3 AIMS OF THE STUDY

Protection against pneumococcal infections early in life is essential, especially in low-income countries. Protection of the very young could be achieved by maternal immunisation or by starting infant vaccinations as early as possible. Pneumococci possess several polysaccharide and protein virulence determinants that contribute to pneumococcal carriage and disease. Certain pneumococcal proteins are common to all pneumococci, regardless of serotype, and new pneumococcal vaccines containing these protein antigens might provide broad protection against pneumococcal infection.

The specific objectives of the present thesis were to evaluate:

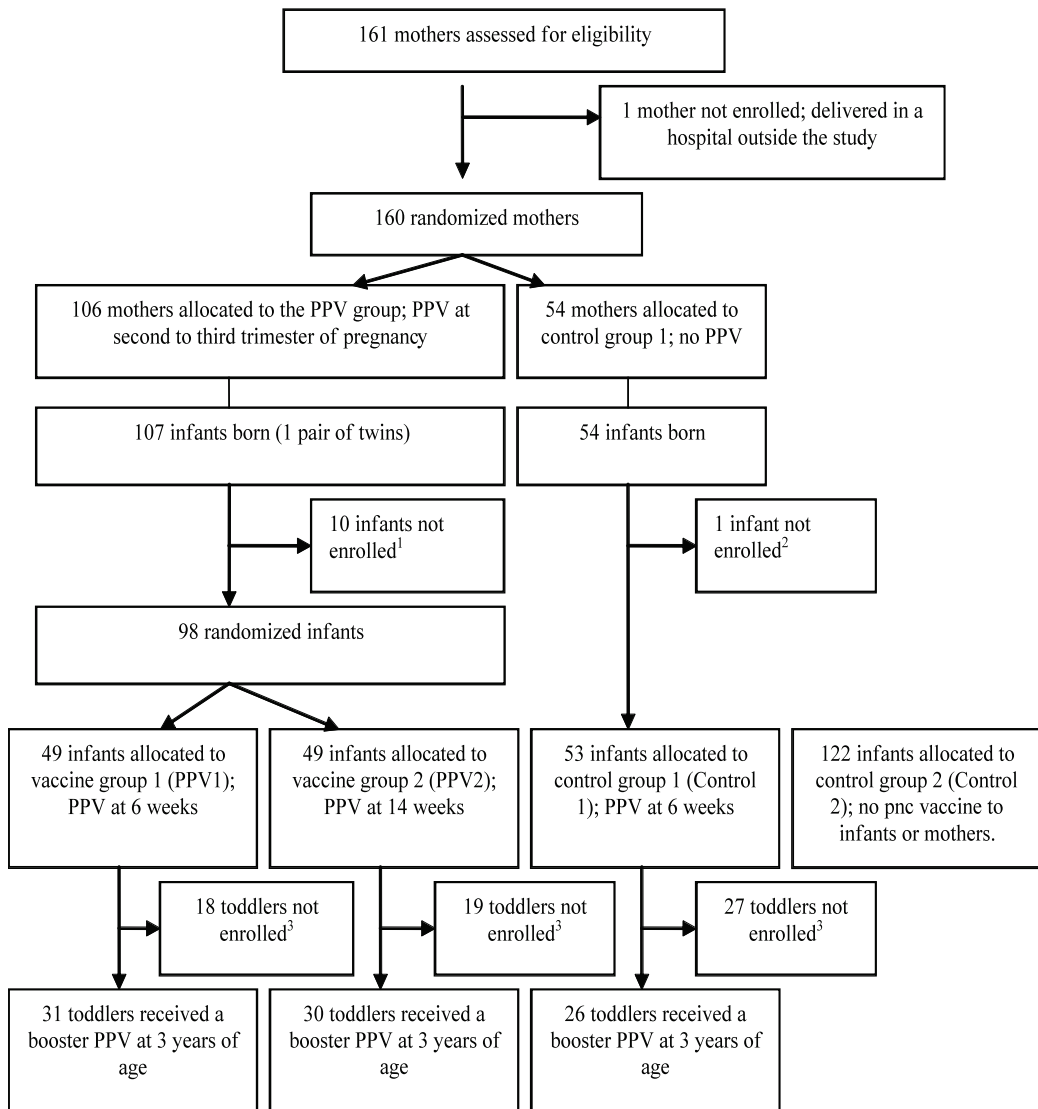
- the persistence of maternal PPV-induced antibodies in the infants, the immunogenicity of PPV in early infancy, the concentrations of anti-polysaccharide antibodies in unimmunised children, the response to a second dose of PPV in toddlers, and the specificity of the polysaccharide antibodies measured by the non-22F enzyme immunoassay (EIA) method (I);
- the transfer of maternal anti-PspA, -PsaA, -Ply, -PspC, -PhtD, -PhtDC and LytC antibodies to the infant, the persistence of maternal anti-protein antibodies in the infant and the development of naturally acquired antibodies to the seven protein antigens in infants (II, III);
- the relation between anti-protein antibody concentrations and pneumococcal carriage in children (II, III).

4 MATERIALS AND METHODS

4.1 Study population, study design, study subjects and vaccines

The maternal immunisation study (MATER) was a randomised, controlled, comparative study that was conducted in 1994–1998 in Cabuyao in the Philippines. Cabuyao is located in the Laguna province, which is a semi-urban, low-to-middle income area south of Manila, where malnutrition is not very common. All pregnant women in their 25th to 33rd week of gestation attending the health centre in Cabuyao who were intending to remain in the area were offered enrolment in the MATER study. Of the 160 enrolled women, 2/3 were randomly assigned to the PPV group (N=106) and 1/3 to the control group 1 (Control 1, N=54) (Fig. 6). A serum sample was obtained upon enrolment from all women, after which the women in the PPV group received PPV containing 25 µg of each of the 23 polysaccharides (Pneumo23, Sanofi Pasteur MSD; former Pasteur Merieux Connaught), the *Haemophilus influenzae* type b (Hib) conjugate vaccine (ActHib, Sanofi Pasteur MSD) and the tetanus vaccine (Tetavax, Sanofi Pasteur MSD). The women in the Control 1 group received only tetanus vaccine. Four weeks later, the women in the PPV group returned for a second visit to the health centre and a second serum sample was taken (N=105). Upon delivery, a cord blood sample was obtained (N=82 and N=42 in the PPV and Control 1 groups, respectively). The safety and immunogenicity results of the mothers have been published previously (Quiambao et al. 2003; Quiambao et al. 2007).

The infants of the mothers in the PPV group were randomly allocated into two subgroups: Pneumococcal Polysaccharide Vaccine Group 1 (PPV1) and Pneumococcal Polysaccharide Vaccine Group 2 (PPV2). The infants of the mothers in the Control 1 group were allocated to Control Group 1 (Control 1) (Fig. 6). The infants in the PPV1 group received PPV at 7 weeks of age and the Hib vaccine at 7 and 12 weeks of age (Table 1). The infants in the PPV2 group received PPV at 17 weeks of age and the Hib vaccine at 12 and 17 weeks of age. The infants in the Control 1 group received PPV at 7 weeks of age and the Hib vaccine at 7, 12 and 17 weeks of age. In addition to the study vaccines, all infants received the regular Filipino Expanded Program of Immunisation (EPI) vaccines at birth and at 7, 12 and 17 weeks of age and at 9 months of age (Table 1). The EPI vaccinations did not change during the MATER study. The results related to the Hib vaccinations have not yet been published and are not included in this thesis.



¹Died before enrolment (5 infants), overage at visit 1 (1 infant), refused blood extraction (1 infant), lost to follow-up (1 infant)

²Lost to follow-up

³Toddlers could not be reached or refused participation

FIGURE 6. Profile of the MATER study (pnc = pneumococcus). Reprinted from (Holmlund et al. 2011) with permission from Elsevier.

The MATER study was further extended with a new group of 122 infants, Control Group 2 (Control 2) (Fig.6). The mothers of these infants were not enrolled in the MATER study. These infants did not receive any pneumococcal vaccine, but they received the Hib vaccine at 12 and 17 weeks of age, as well as the EPI vaccines (Table 1).

TABLE 1. Schedule for the expected and observed average ages of the infants at each visit. The observed age will be used in this thesis (please see section 4.2.1). The schedule also shows the vaccines received by the different study groups at each visit. The mothers of the PPV1 and PPV2 infants were vaccinated with PPV during pregnancy, while the mothers of the Control 1 and Control 2 infants were not. See Fig. 6 for a definition of the study groups PPV1, PPV2, Control 1 and Control 2. PPV = 23 valent pneumococcal polysaccharide vaccine, Hib = *Haemophilus influenzae* type b conjugate vaccine. The regular Filipino Expanded Program of Immunisation (EPI) vaccines given at the different visits were: Visit 1: Hepatitis B (HBV), Diphtheria, Tetanus, Pertussis (DTwP), Polio (OPV); visit 2: HBV, DTwP, OPV; visit 3: HBV, DTwP, OPV; visit 5: Measles

	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7/8
Expected age	6 wk	10 wk	14 wk	18 wk	9 mo	10 mo	≥2 y (pre/post)
Observed age	7 wk	12 wk	17 wk	22 wk	9 mo	10 mo	3 y (pre/post)
PPV1	PPV, Hib, EPI	Hib, EPI	EPI	-	EPI	-	PPV, Hib
PPV2	EPI	Hib, EPI	PPV, Hib, EPI	-	EPI	-	PPV, Hib
Control 1	PPV, Hib, EPI	Hib, EPI	Hib, EPI	-	EPI	-	PPV, Hib
Control 2	EPI	Hib, EPI	Hib, EPI	-	EPI	-	-

The infants in the PPV1, PPV2 and Control 1 groups were recruited again at three years of age for a second dose of PPV (N=31, N=30, N=26 for PPV1, PPV2 and Control 1, respectively). They also received an additional dose of the Hib vaccine (Fig. 6, Table 1).

The MATER study was conducted over different periods of time. In July 1994, the first pregnant women were enrolled and the samples from the PPV1, PPV2 and Control 1 infants were collected until November 1996. In February 1996, the Control 2 infants were enrolled. The last samples from the Control 2 infants were taken in March 1998. In February 1998, a second dose of PPV was given to the PPV1, PPV2 and Control 1 toddlers. The last samples from the toddlers were taken in July 1998.

4.2 Clinical samples and bacteriological methods

4.2.1 Serum samples

The clinical samples were collected as described in detail in study I of this thesis. Serum samples were scheduled to be obtained from the infants at 6 weeks (visit 1), 10 weeks (visit 2), 14 weeks (visit 3) and 18 weeks of age (visit 4), as well as at the ages of 9 and 10 months (visits 5 and 6). Serum samples from the infants receiving a second dose of PPV were scheduled to be obtained at ≥ 2 years of age before vaccination (visit 7) and 1 month after vaccination (visit 8). The real ages at the time of samplings were on average 7, 12, 17, 22, 40 and 45 weeks (9 and 10 months, respectively) and 3 years (Table 1). The real ages are used in this thesis.

All samples from the mothers and the children were collected at the health centre in Cabuyao in the Philippines, with the exception of the cord blood samples, which were mostly taken at home (since most of the children were born at home). The cord blood samples were kept in the refrigerator and after an average of 6 h (max 24 h), the samples were centrifuged. All serum samples were stored at -20°C and sent frozen to the National Public Health Institute, Helsinki, Finland (currently: the National Institute for Health and Welfare), where they were analysed.

4.2.2 Nasopharyngeal samples and bacterial cultures

At every visit, when a serum sample was taken, a nasopharyngeal swab for the detection of pneumococcal carriage was also collected from the infants, except at three years of age. Nasopharyngeal samples were obtained via the nasal route at 7, 12, 17 and 22 weeks of age, as well as at 9 and 10 months of age, by the study nurse. Pneumococcal carriage was defined as a nasopharyngeal culture positive for *S. pneumoniae*. The nasopharyngeal swabs were cultured on agar plates. If the growth on the plate was pure and typical of *S. pneumoniae*, only one colony was picked and subjected to standard identification tests. If there were alpha haemolytic streptococci colonies with different colony morphology, 3–5 colonies were picked. A more thorough description is given in study II of this thesis. The information on the serotypes carried is not included in this thesis.

Definitions (studies II and III)

The infant samples were grouped into two categories according to the pneumococcal carriage status of the infant (Pnc+/Pnc-) at each age point. The age points are listed

in Tables 2 and 3. The antibody concentrations were examined separately for both groups.

Carriers (Pnc+):

Infants with at least one nasopharyngeal culture positive for *S. pneumoniae* up to the age of sampling in question. Once the infant had become a carrier (Pnc+), he/she stayed in the Pnc+ group, although he/she did not have to be a carrier at every following visit.

Non-carriers (Pnc-):

Infants with no nasopharyngeal cultures positive for *S. pneumoniae* up to the age of sampling in question.

4.3 Subsets of the MATER study

Study I

In 1995–1999, the anti-polysaccharide antibodies to serotypes 1, 5, 6B, 14, 18C and 19F in all samples of the PPV1, PPV2 and Control 1 mothers and infants, as well as in the 7 weeks, 22 weeks and 9 months samples of the Control 2 infants were analysed at the National Public Health Institute, Helsinki, Finland, by EIA without 22F-polysaccharide neutralisation (non-22F EIA). In 2003–2004, the anti-polysaccharide antibodies to the above-mentioned serotypes, except for serotype 18C, were reanalysed at the National Public Health Institute, Helsinki, Finland, in the 7, 12, 17 (only PPV 2 infants) and 22 weeks, as well as in the 10 months samples (or 9 months sample, if the 10 months sample was finished) of the first 33–34 infants from the PPV1, PPV2 and Control 1 groups with the improved EIA method employing 22F-polysaccharide neutralisation (22F EIA). All serum samples of the Control 2 infants (except the 9 months sample, unless the 10 months sample was finished) were analysed with the 22F EIA. Serotype 18C was not reanalysed with the 22F EIA method for financial reasons and because 18C may be the least common of these serotypes in Filipino infants (Lankinen et al. 1994).

Study II

In 2001, anti-protein antibodies to PspA, PsaA and Ply were analysed by EIA in all serum samples obtained at the six first visits from the Control 1 and Control 2 infants (N=173). Since the age range of the infants at each visit was wide and since the actual age is important when analysing naturally acquired antibodies, the samples were regrouped into nine new groups according to the observed age instead of the original six groups based on the scheduled visits (Table 2).

TABLE 2. In study II, the serum samples from the Control 1 and Control 2 infants were grouped into nine age groups according to the observed biological age of the infant at the time of serum sample collection. The average age (in weeks) and number of samples within each age group are shown.

Age group (wk)	Average age within group (wk)	No. of infant samples (n = 935)
< 8	7	132
8 to 11	10	106
12 to 15	14	136
16 to 19	18	128
20 to 23	22	116
24 to 37	27	25
38 to 41	40	131
42 to 45	44	125
≥ 46	48	36

Study III

In 2004, anti-protein antibodies to PspC, PhtD, PhtDC and LytC in serum samples of the Control 1 infants were analysed by EIA. Since there were fewer samples in this study than in Study II, the samples were regrouped into eight groups according to the observed age (Table 3).

TABLE 3. In study III, the serum samples from the Control 1 infants were grouped into eight new age groups according to the observed biological age of the infant at the time of serum sample collection. The average age (in weeks) and the number of samples within each age group are shown.

Age group (wk)	Average age within group (wk)	No. of infant samples (n = 263)
< 8	7	42
8 to 11	11	34
12 to 15	14	40
16 to 19	18	32
20 to 28	22	33
37 to 41	40	41
42 to 45	44	28
≥ 46	49	13

4.4 Serological methods

4.4.1 Antigens used for the measurement of serum antibodies

Polysaccharides (PS):

Capsular PSs of pneumococcal serotypes 1, 5, 6B, 14, 18C, 19F and 22F were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The CPS used for absorption of the anti-CPS antibodies was obtained from Statens Serum Institut (Copenhagen, Denmark).

PspA:

The PspA antigen was received from Dr Robert Becker at Aventis Pasteur, Swiftwater, PA, USA. A recombinant family 1, clade 2 PspA from the Rx1 strain comprising the first 315 amino acids of the amino-terminal part was used for the detection of anti-PspA antibodies.

PsaA:

The recombinant PsaA antigen produced in the QiaexpressTM vector (Qiagen, Inc. Chatsworth, CA) was kindly provided by Dr Jacquelyn Sampson, Centers for Disease Control and Prevention (CDC), Atlanta, USA. The *psaA* gene from serotype 2, strain D39 had been cloned into the pQE30 plasmid and transferred into *Escherichia coli*, and from there the his-tagged PsaA had been purified by the use of Ni-NTA chromatography (Pilling et al. 1998).

Ply:

The Ply mutant PdB was received from Prof. James Paton, Adelaide University, Australia. The PdB recombinant protein with a W433F point mutation had been produced in *E. coli*. This mutation reduces the haemolytic activity of the toxin, but does not reduce its antigenicity (Paton et al. 1991).

PspC:

The recombinant PspC protein used was 446 amino acids long and had a size of 50.6 kD. The *pspC* gene was from serotype 2, strain D39 and the antigen had been produced in *E. coli*. The PspC antigen did not contain the choline-binding domain of the protein thought to be non-immunogenic (Gosink et al. 2000). This antigen was received from Glaxo Smith Kline (GSK).

PhtD and PhtDC:

The genes for the PhtD and PhtDC proteins were isolated from the serotype 4 Norway strain and the recombinant antigens had been produced in *E. coli*. The PhtD antigen was 819 amino acids long and had a size of 91.6 kD.

The recombinant PhtDC represents the putatively exposed and protection-eliciting carboxy-terminal fragment of the PhtD protein. The used PhtDC antigen contained amino acids 383–839 and was about half of the size of the PhtD antigen (52.3 kD). These antigens were received from GSK.

LytC:

The recombinant LytC antigen used was 218 amino acids long and had a size of 25.8 kD. The antigen lacked the choline-binding domain of the protein, which has been suggested to be neither protective nor immunogenic in a mouse model of sepsis (Wizemann et al. 2001). The gene was from the serotype 4 Norway strain and the protein had been produced in *E. coli*. This antigen was received from GSK.

4.4.2 EIA for measurement of the serum antibodies to polysaccharide and protein antigens**Non-22F EIA (I)**

The concentrations of the serum IgG anti-polysaccharide antibodies to serotypes 1, 5, 6B, 14, 18C and 19F were measured by the non-22F EIA as described earlier (Käyhty et al. 1995). As a reference serum, the commercial reference serum 89-SF was used (Quataert et al. 1995). The lower limits of quantitation of the non-22F EIA for serotypes 1, 5, 6B, 14, 18C and 19F were 0.10, 0.15, 0.09, 0.14, 0.07 and 0.17 µg/ml, respectively. The polysaccharide results of this thesis were received by the non-22F method, except for the results obtained by the 22F method in Fig. 9.

22F EIA (I)

Since the non-22F EIA measures unspecific antibodies at low concentrations of naturally acquired antibodies (Concepcion et al. 2001; Soininen et al. 2002; Inostroza et al. 2005; Henckaerts et al. 2006), the improved EIA method with 22F inhibition (Simell et al. 2008) was used to re-analyse the IgG antibody concentrations to serotypes 1, 5, 6B, 14 and 19F in a subset of PPV1, PPV2 and Control 1 infants, and to all anti-polysaccharide antibody measurements of the Control 2 infants (see section 4.3). As a reference serum, the commercial reference serum 89-SF was used (Quataert et al. 1995; Quataert et al. 2004). The lower limits of quantitation of the 22F EIA for serotypes 1, 5, 6B, 14 and 19F were 0.07, 0.09, 0.06, 0.12 and 0.13 µg/ml, respectively.

PspA, PsaA and Ply EIA (II). The concentrations of the serum IgG antibodies to PspA, PsaA and Ply were measured with an EIA method described earlier (Rapola et al. 2000) with minor modifications: The wells of the microtiter plates were coated with the PsaA antigen at +4°C overnight and the lower limits of quantitation were revised for the present study: PspA 1.60 U/ml, PsaA 2.91 U/ml and Ply 0.84 U/ml. Antibody concentrations below the lower limit of quantitation were assigned values equivalent to half of the lower quantitation limit. The anti-PspA, -PsaA and -Ply concentrations are given as units per millilitre (U/ml) and cannot therefore be compared to each other or to the anti-protein antibody concentrations reported in other studies.

PspC, PhtD, PhtDC and LytC EIA (III). The concentrations of the serum IgG antibodies to PspC (CbpA), PhtD, PhtDC and LytC were measured using the EIA method described in Study III. As a reference serum, a commercial human serum was used (Lucron H006W11, Lucron Bioproducts, De Pinte, Belgium) for which the anti-PspC, -PhtD, -PhtDC and -LytC IgG concentrations had been previously determined to be 23.49 µg/ml, 12.3 µg/ml, 2.99 µg/ml and 3.89 µg/ml, respectively. Antibody concentrations below the lower limit of quantitation (0.16 µg/ml for PspC, 0.11 µg/ml for PhtD, 0.07 µg/ml for PhtDC and 0.41 µg/ml for LytC) were assigned values equivalent to half of the lower quantitation limit.

4.5 Sample size, thresholds and statistical methods

Sample size:

The sample size of this study was chosen by convenience with the immunogenicity as the main objective driving the size of the study. No formal sample size calculations were made. Based on earlier findings, approximately 50–60 subjects per group was considered sufficient to observe significant differences in antibody concentrations.

Thresholds:

The threshold concentration of 0.35 µg/ml has been associated with protection against pneumococcal invasive disease (WHO 2009) and the more robust threshold of 1 µg/ml might be associated with protection against pneumococcal pneumonia (Örtqvist et al. 2007).

Statistical methods in study I:

In some study groups, especially in the samples taken close to 10 months of age, up to 82% of the anti-polysaccharide concentrations were below the lower limit of

quantitation, i.e. undetectable. Assigning half of the limit of quantitation value to up to 82% of the samples would have reduced the variation of the data, which in turn would have had an effect on the geometric mean concentrations (GMCs), confidence intervals (CIs) and statistical significances. Therefore, the data were modelled as left censored. Thus, the values below the limit of quantitation were assumed to be part of the log-normal distribution, i.e. the log-normal curve continues below the limit of quantitation.

For modelling, the censored normal regression function of Stata 9.2 (StataCorp, 4905 Lakeway Drive College Station, Texas) was used. The different study groups were different factors in the model. The model did not give any exact values to the data that were below the limit of quantitation, but used the information of the left-censored log-normal curve to calculate the GMCs and the 95% CIs. For all the data that were above the limit of quantitation, the model used the measured values. When the degree of left censoring was small, the traditionally calculated GMC and their CIs were close to those given by the model. Therefore, all data from Study I were analysed by the model, irrespective of the number of samples below the detection limit.

The statistical significances of the anti-polysaccharide comparisons were based on the 95% CI of the geometric mean ratios (GMRs) received by the censored normal regression model, giving a p-value that is <0.05 . If 1 was not included in the CIs, the difference was significant.

The responses to the second dose of PPV were compared between the PPV1, PPV2 and Control 1 groups to find out whether maternal or early infant vaccinations would have affected the responses to the second dose of PPV. The differences between the three toddler groups are expressed as GMRs with 95% CIs. In addition to the 95% CIs, the Holm method was used to analyse in which groups the responses differed significantly. The Holm method contains multiplicity adjustments, i.e. makes adjustments to decrease chance as the cause of the significant findings.

Study II and III: The data were log transformed and the concentrations were summarised as GMCs with 95% CIs. The significance of the differences in anti-PsaA concentration between the mothers and the infants was based on the 95% CIs of the GMCs. At the time points when the CIs did not overlap, the difference was significant ($p<0.05$).

This thesis: The GEE logistic marginal regression model with unstructured correlation structure between different time points was used to examine whether the odds ratio to carry pneumococcus differed at the different time points between the PPV1, PPV2, Control 1 and Control 2 infants (i.e. time and group interaction). The

same model was also used to examine the differences in odds to carry pneumococci in the different study groups. The statistical significances are based on p-values and on the 95% CIs of the odds ratios.

4.6 Ethical aspects

Written informed consents were obtained from all the mothers before enrolment. The ethical approval of the MATER study with its extensions, as well as the extension for the measurement of anti-protein antibodies, was granted by the Institutional Review Board of the Research Institute for Tropical Medicine (RITM), the research arm for infectious diseases of the Philippine Department of Health. Since this clinical trial was carried out (1994–1998) before the clinical trial registers existed, this study does not have a register number for clinical trials.

5 RESULTS

5.1 Transfer of the maternal anti-polysaccharide antibodies and their persistence in infants (I)

In the MATER study the maternal anti-polysaccharide antibodies have previously been reported to be transferred from the mothers to their infants (Quiambao et al. 2003; Quiambao et al. 2007). In this thesis, we wanted to evaluate the persistence of these maternal anti-polysaccharide antibodies in their infants. To accomplish this, the data for serotypes 6B, 14, 18C and 19F were used, because the infants did not have an antibody production of their own to these serotypes in response to PPV (see Fig. 7). After birth, the anti-polysaccharide antibody concentrations of the infants in all study groups started to decrease due to the waning of the maternal antibodies. The mean antibody concentrations remained significantly higher in infants of the PPV-vaccinated mothers (PPV1 and PPV2) for about 4–5 months as compared to the infants of the unvaccinated mothers (Control 1) (Fig. 7).

5.2 Polysaccharide antibodies in infants (I)

5.2.1 PPV-induced and naturally acquired antibodies in infants

The PPV1 and Control 1 infants received PPV at 7 weeks of age and the PPV2 infants at 17 weeks of age. The pre-vaccination anti-polysaccharide GMCs in the PPV1 infants were higher compared to the Control 1 infants, due to the higher concentrations of maternal antibodies. The pre-vaccination GMCs in the PPV1 infants were also higher than in the PPV2 infants, since the concentrations of maternal antibodies had declined more in the PPV2 infants by the time of vaccination, which occurred 10 weeks later than in the PPV1 infants (Fig. 7).

In the PPV2 and Control 1 infants, PPV induced an increase in antibody concentrations to serotypes 1 and 5, but not to serotypes 6B, 14, 18C and 19F (Fig. 7). Probably due to the lower pre-vaccination concentration of the Control 1 and PPV2 infants, the antibody concentrations to serotypes 1 and 5 increased in the Control 1 and PPV2 infants after immunisations at 7 and 17 weeks, respectively, whereas this was not seen in the PPV1 infants with higher concentrations of maternal antibodies. Although the anti-1 and anti-5 antibody concentrations increased in the Control 1 infants (and not in the PPV1 infants), the post-vaccination

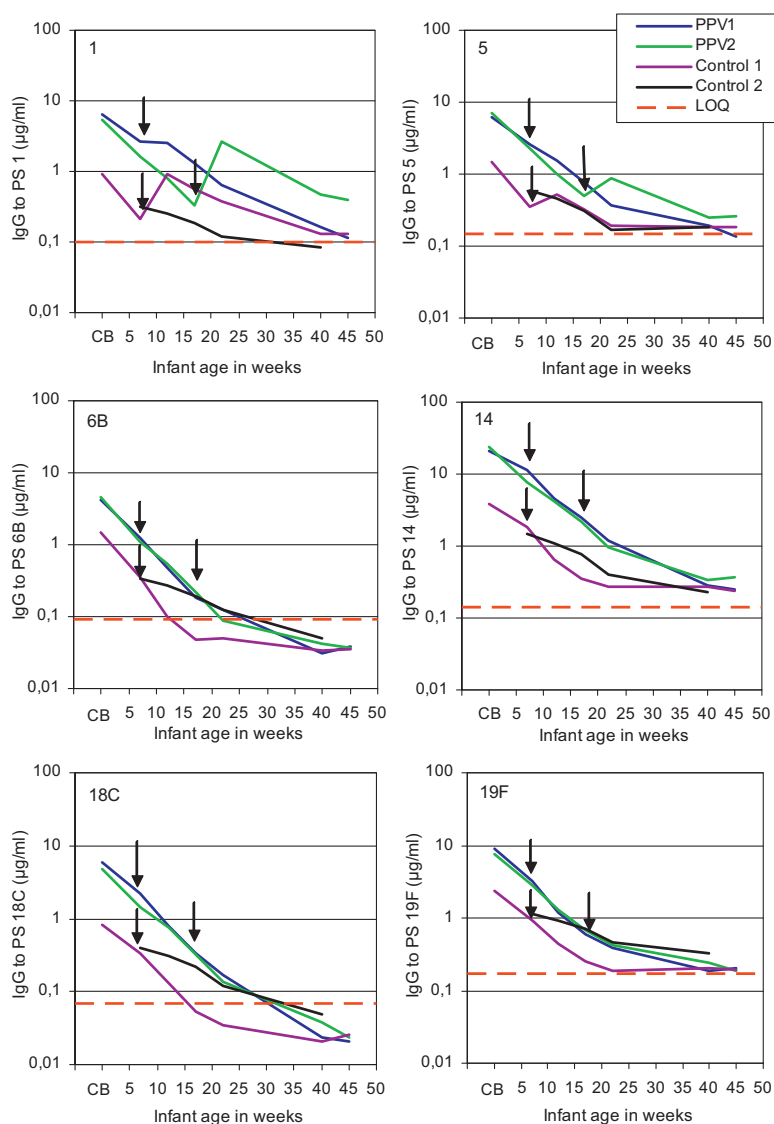


FIGURE 7. The kinetics of the serotype-specific anti-polysaccharide antibody concentrations to serotype 1, 5, 6B, 14, 18C and 19F as measured by non-22F EIA in the PPV1 (blue), PPV2 (green), Control 1 (purple) and Control 2 (black) infants. PPV1 and Control 1 infants received PPV at 7 weeks and PPV2 infants at 17 weeks of age (vaccinations indicated by arrows). Control 2 infants did not receive any pneumococcal vaccines. The geometric mean concentrations ($\mu\text{g/ml}$) in the cord bloods (CB) and in the consecutive sera taken at 7, 12, 17 and 22 weeks and at 9 and 10 months of age are shown. For Control 2 infants, only three samples were analysed: 7 and 22 weeks and 9 months. The number of samples: $N=40\text{--}42$ for cord blood, $N=44\text{--}53$ for PPV1, PPV2 and Control 1 infants and $N=107\text{--}122$ for Control 2 infants. LOQ = lower limit of quantitation. Reprinted from (Holmlund et al. 2011) with permission from Elsevier.

GMCs of antibodies to serotypes 1 and 5 were higher in the PPV1 infants than in the Control 1 infants (Fig. 7). In the PPV2 infants, the post-vaccination concentrations to serotype 1 were similar as compared to the PPV1 infants, but the antibody concentration to serotype 5 was lower in the PPV2 infants. For serotypes 6B, 14, 18C and 19F, the post-vaccination concentrations decreased compared to the pre-vaccination concentrations (Fig. 7).

The anti-polysaccharide antibody concentrations to serotypes 1, 5, 6B, 14, 18C and 19F of the unimmunised infants of the unimmunised mothers (Control 2) decreased with age and remained low until the end of the follow-up (Fig. 7). An unexpected finding was that the immunised infants of unimmunised mothers (Control 1) had lower GMCs than the unimmunised infants of unimmunised mothers (Control 2) for serotypes 6B, 18C and 19F (Fig. 7). The differences were significant at 22 weeks for serotypes 6B, 18C and 19F and at 9 months for serotypes 18C and 19F.

In general, the anti-polysaccharide antibody concentrations for all serotypes decreased with increasing age in all study groups, with the exception of the responses to PPV (Fig. 7). At 9 months of age, all study groups had similar GMCs of anti-polysaccharide antibodies, regardless of the previous vaccinations. Serotype 1, however, was an exception: The GMCs of anti-1 antibodies were significantly higher in the vaccinated infants (PPV1, PPV2 and Control 1) as compared to the unvaccinated infants (Control 2) (Fig. 7).

5.2.2 The proportions of vaccine-induced anti-polysaccharide antibodies exceeding the threshold of 0.35 µg/ml or 1 µg/ml in infants

The percentage of the PPV1, PPV2 and Control 1 infants with antibody concentrations to polysaccharides 1, 5, 6B, 14, 18C and 19F higher than 0.35 µg/ml or 1 µg/ml at the six study visits are shown in Fig. 8. At 7 weeks of age (visit 1), 82–98% of the infants of the PPV-immunised mothers (PPV1 and PPV2) had antibody concentrations to all six serotypes that were higher than 0.35 µg/ml, whereas 58–94% had antibody concentrations higher than 1 µg/ml. The corresponding percentages of infants of unimmunised mothers (Control 1) were 40–83% and 13–66% for the thresholds 0.35 µg/ml and 1 µg/ml, respectively (Fig. 8).

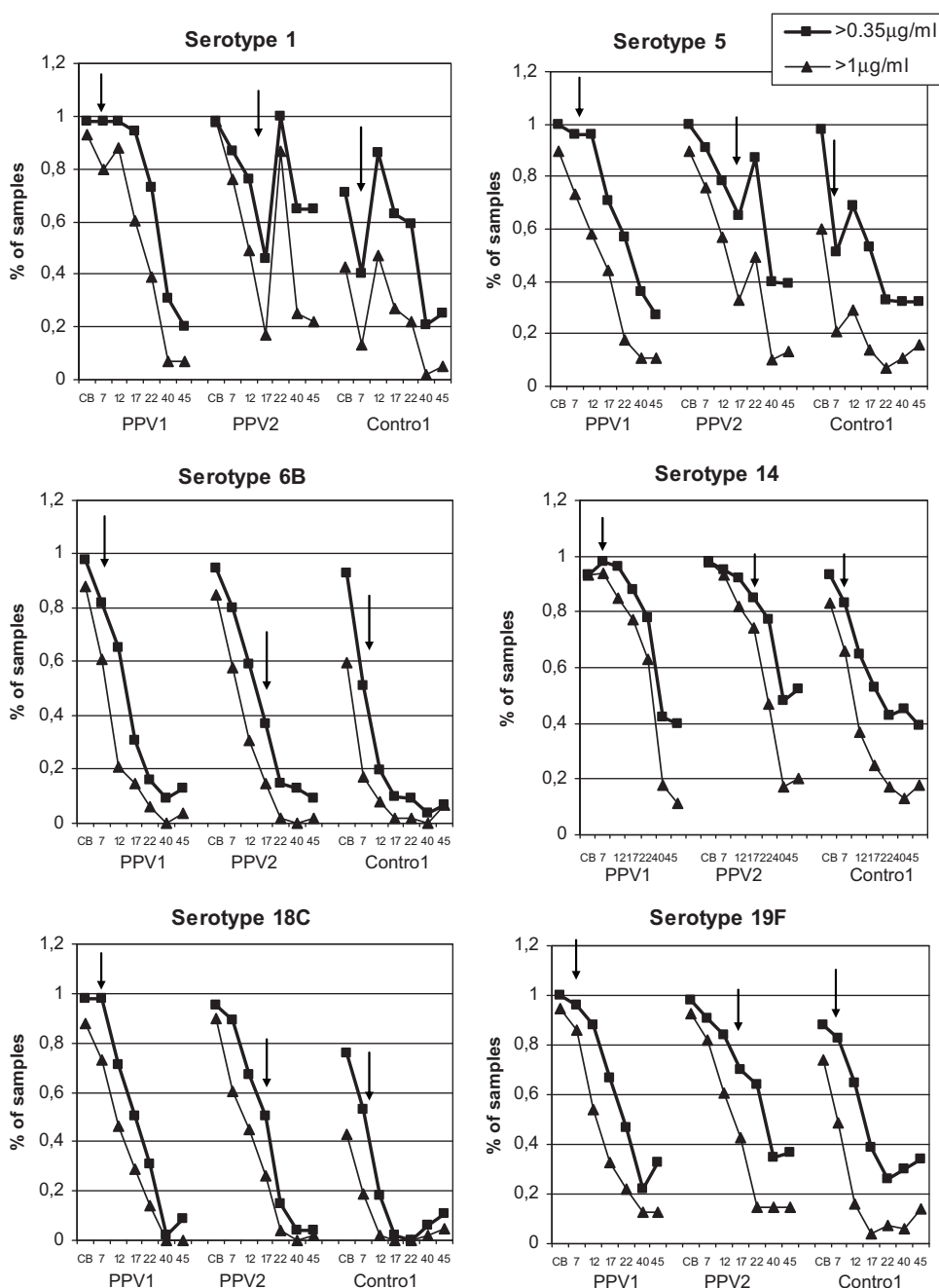


FIGURE 8. Percentages of the PPV1, PPV2 and Control 1 infant sera with an antibody concentration higher than $0.35\mu\text{g/ml}$ or $1\mu\text{g/ml}$ to serotypes 1, 5, 6B, 14, 18C and 19F. Reprinted from (Holmlund et al. 2011) with permission from Elsevier.

The post-vaccination antibody concentrations to serotypes 1 and 5 were higher than 0.35 µg/ml in most (96–98%) of the PPV1 infants and higher than 1 µg/ml in 58–88% of the sera of the Control 1 infants (Fig. 8). In the PPV2 infants, the corresponding percentages of the post-vaccination concentrations to serotypes 1 and 5 higher than 0.35 µg/ml were 87–100% and higher than 1 µg/ml were 49–87%. Among the Control 1 infants, 69–86% had concentrations higher than 0.35 µg/ml and 29–47% had concentrations higher than 1 µg/ml. In spite of the PPV vaccination, the percentage of samples above these thresholds decreased with age for serotypes 6B, 14, 18C and 19F as the maternal antibodies disappeared (Fig. 8).

At the age of 9–10 months (i.e. at visits 5 and 6), the infants had started to produce their own naturally induced anti-polysaccharide antibodies, as seen by the slight increase in the percentages of the serotype-specific antibody concentrations exceeding the indicated thresholds (Fig. 8). At 9–10 months, 20–65% of the infants had concentrations higher than 0.35 µg/ml and 2–25% higher than 1 µg/ml for serotypes 1, 5, 14 and 19F. For serotypes 6B and 18C, the percentages of infants with concentrations higher than 0.35 µg/ml or 1 µg/ml were smaller: 2–13% and 0–7%, respectively (Fig. 8).

5.2.3 Response to the second dose of PPV in toddlers

Of the total of 151 PPV1, PPV2 and Control 1 infants, 87 (58%) children were reached for a second dose of PPV at the age of three years (Fig. 6). The GMCs of the anti-polysaccharide antibodies of the toddlers were similar before vaccination regardless of the previous PPV vaccinations during early infancy (Table 4). The toddlers responded well to the second dose of PPV (Table 4) and the post-vaccination GMCs were significantly greater than the pre-vaccination GMCs, for all serotypes except for 6B. The GMRs varied from 3 to 11 for serotypes 5, 6B, 14 and 19F, and from 17 to 76 for serotypes 1 and 18C (Table 4). The significant differences seen between some of the study groups indicated by an asterisk in Table 4 were omitted after analyses with the Holm method, except for the difference between PPV1 and PPV2 for serotype 1 ($p=0.0000$).

Before vaccination, 0–57% of the infants had an antibody concentration higher than 0.35 µg/ml and 0–30% higher than 1 µg/ml. After the second dose of PPV, 86–100% of the infants had antibody concentrations higher than 0.35 µg/ml and 61–100% higher than 1 µg/ml to serotypes 1, 5, 18C and 19F. For serotypes 6B and 14, the corresponding percentages were 54–86% and 21–69%, respectively. In general, serotype 6B was found to induce the lowest mean antibody concentrations.

TABLE 4. Responses measured by non-22F EIA to a second dose of PPV at three years of age among 87 children. Serotype-specific (1, 5, 6B, 14, 18C and 19F) geometric mean antibody concentrations (GMCs) and 95% confidence intervals (CIs) in toddlers pre- and post-PPV are shown. Furthermore, the geometric mean ratios (GMRs) for the post- and pre-immunization concentrations are provided. The percentages of infants with a pre- and post-immunization anti-polysaccharide concentration higher than 0.35 µg/ml and 1 µg/ml are given. The numbers of pre- and post-immunization samples are 29-30/28-29, 30/28-29 and 26/24 for the PPV1, PPV2 and Control 1 infants, respectively. Modified from (Holmlund et al. 2011).

Pnc type	PPV1				PPV2				Control 1			
	GMC (95% CI)	GMR	% > 0.35	% > 1	GMC (95% CI)	GMR	% > 0.35	% > 1	GMC (95% CI)	GMR	% > 0.35	% > 1
1	Pre	0.10 (0.05-0.15)	0	0	0.19 (0.10-0.28)	23*	27	3	0.16 (0.08-0.24)	39**	23	12
	Post	8.48 (5.78-11.18)	76	100	4.66 (3.18-6.14)		100	100	6.56 (4.26-8.85)		100	100
5	Pre	0.37 (0.21-0.53)	50	20	0.42 (0.24-0.60)	6*	50	13	0.40 (0.22-0.58)		50	19
	Post	4.22 (2.62-5.83)	11	100	2.67 (1.66-3.69)		97	83	2.83 (1.65-4.02)	7	96	92
6B	Pre	0.14 (0.07-0.21)	30	3	0.17 (0.09-0.25)	5	33	7	0.11 (0.05-0.17)		23	0
	Post	0.47 (0.17-0.76)	4	55	0.76 (0.28-1.24)		71	39	0.34 (0.10-0.57)	3	54	21
14	Pre	0.16 (0.06-0.26)	23	13	0.44 (0.19-0.70)	6	57	27	0.16 (0.06-0.27)		35	8
	Post	0.77 (0.30-1.25)	5	69	2.51 (1.00-4.02)		86	69	1.25 (0.42-2.08)	8	75	63
18C	Pre	0.07 (0.01-0.12)	27	10	0.10 (0.02-0.17)	21	17	3	0.11 (0.02-0.20)		19	15
	Post	3.29 (1.84-4.74)	39	97	2.37 (1.33-3.42)		97	72	2.28 (1.18-3.39)	17**	100	88
19F	Pre	0.29 (0.11-0.47)	55	28	0.33 (0.13-0.52)	7	53	30	0.38 (0.14-0.61)		50	27
	Post	1.40 (0.76-2.04)	4	86	2.45 (1.34-3.55)		96	86	1.36 (0.89-2.04)	3	88	63

* The GMRs (PPV2:PPV1) are significantly different at a 5% level

** The GMCs (Control 1:PPV1) are significantly different at a 5% level

5.3 Comparison of the anti-polysaccharide antibody data obtained by non-22F EIA and 22F EIA (I)

The original determinations of the anti-polysaccharide antibody concentrations were performed with the non-22F EIA. Since the methodology has developed since then, we wanted to compare the results obtained by the non-22F EIA and the more specific 22F-EIA in sets of sera to find out whether the different methods would affect the interpretation of the results. The 22F EIA analyses were done for a subset of infant sera (see section 4.3). The two EIA methods gave similar results for the sera of infants of immunised mothers (PPV1 and PPV2) (Fig. 9), indicating that the infants had received serotype-specific vaccine-induced antibodies from their mothers. The antibody concentrations obtained by the 22F EIA and non-22F EIA methods to serotypes 1, 5 and 6B in the infants of the unimmunised mothers (Control 1 and Control 2) varied somewhat more during the first months of life. In the Control 1 infants, the differences were seen until the age of approximately 7 weeks, when they were immunised, whereas in the Control 2 infants the differences were seen until the age of approximately 17–22 weeks.

The finding that the Control 1 infants had significantly lower antibody concentrations than the Control 2 infants for serotypes 6B, 18C and 19F when measured by non-22F EIA could not be confirmed with the 22F EIA method (Fig. 9). Based on the more specific results obtained by the 22F EIA, this suggests that PPV given in early infancy did not decrease the antibody concentrations in the immunised infants compared to the unimmunised infants.

5.4 Transfer of naturally acquired maternal anti-protein antibodies and their persistence in infants (II-III)

The naturally acquired maternal anti-protein antibodies were transferred from the mothers to the infants. The anti-protein antibody concentrations in the cord blood samples were similar to those of the mothers (Fig. 10 and 11). Depending on the protein, the maternal anti-PspA, -Ply, -PspC, -PhtD, -PhtDC and -LytC antibodies persisted in the infants for at least 18–22 weeks (i.e. 4–5 months). The persistence of the maternal anti-PsaA antibodies could not be determined, since the production of the infants' own anti-PsaA antibodies started early.

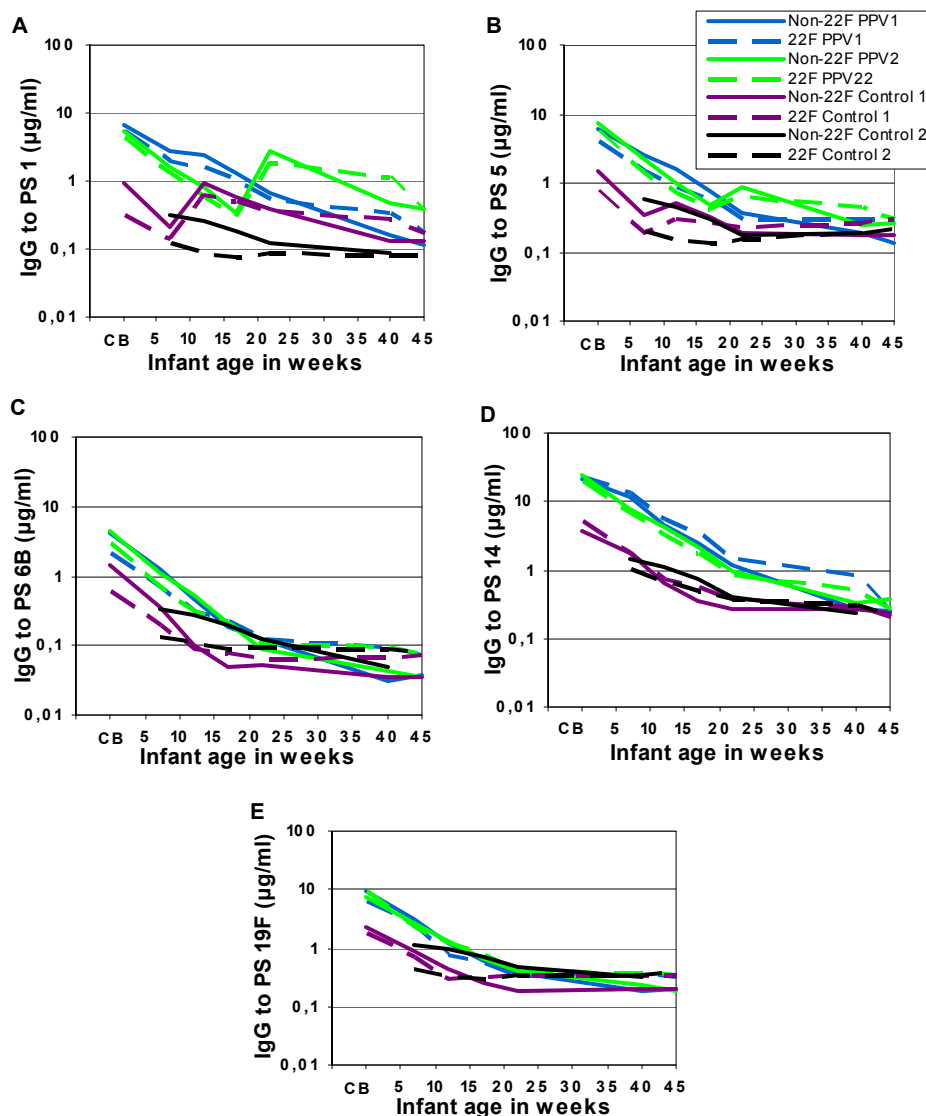


FIGURE 9. Kinetics of the serotype-specific anti-polysaccharide antibody concentrations to serotypes 1 (A), 5 (B), 6B (C), 14 (D) and 19F (E) as measured by non-22F EIA (solid lines) and 22F EIA (dashed lines) in the PPV1 (blue), PPV2 (green), Control 1 (purple) and Control 2 (black) infants. Number of samples in non-22F: N=40–53 for PPV1, PPV2 and Control 1 and N=107–122 for Control 2. Number of samples in 22F EIA: N=20–34 for PPV1, PPV2 and Control 1, and N=109–120 for Control 2. CB = cord blood. Modified from (Holmlund et al. 2011).

5.5 Naturally acquired anti-protein antibodies in infants (II and III)

5.5.1 Naturally acquired anti-protein antibodies in infants

The kinetics of the anti-PsaA antibodies differed from that of the other anti-protein antibodies. The synthesis of the infants' own anti-PsaA antibodies was detected already after 7 weeks of age, and already at 14 weeks of age, the GMC of anti-PsaA antibodies in the infants reached the anti-PsaA GMC of the mothers. The anti-PsaA concentrations of the infants continued to increase and were significantly higher than in the mothers from 5 to 10 months (22–44 weeks) of age (Fig. 10). The

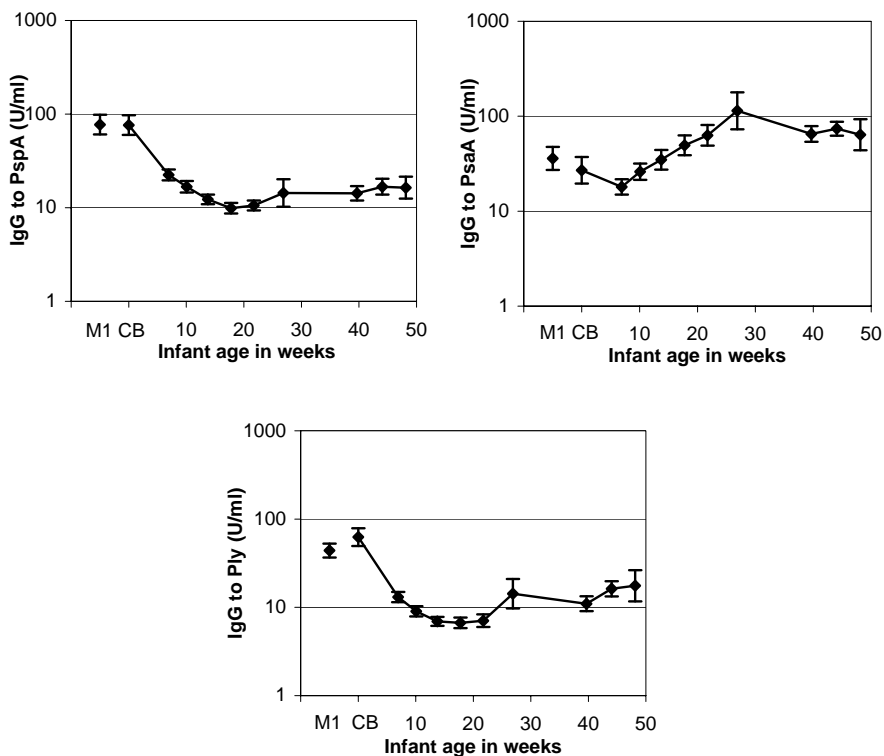


FIGURE 10. The kinetics of the development of the naturally acquired antibodies to pneumococcal proteins PspA, PsaA and Ply. The geometric mean concentration (U/ml) and the 95% confidence intervals of anti-PspA (left), -PsaA (right) and -Ply (bottom) antibodies in sera of mothers (M1) taken during the second or third trimester of pregnancy, in the cord blood (CB) and in the consecutive sera of infants (see Table 2). The number of samples at each time point was as follows: N=51 (M1), 39 (CB), 131–132 (7 weeks), 106 (10 weeks), 136 (14 weeks), 128 (18 weeks), 114–116 (22 weeks), 25 (27 weeks), 130–131 (40 weeks), 125 (44 weeks) and 35–36 (48 weeks). Modified from (Holmlund et al. 2006).

production of naturally induced anti-PspA, -Ply, -PspC, -PhtD, -PhtDC (the carboxy-terminal end of PhtD), and -LytC antibodies started much later; after 18–22 weeks of age (i.e. 4–5 months) (Fig. 10 and 11). At the end of the follow-up, the anti-protein antibody concentrations (except anti-PsaA) of the infants were still lower than those of the mothers, with the exception of the anti-LytC concentrations, which were similar to those in the mothers.

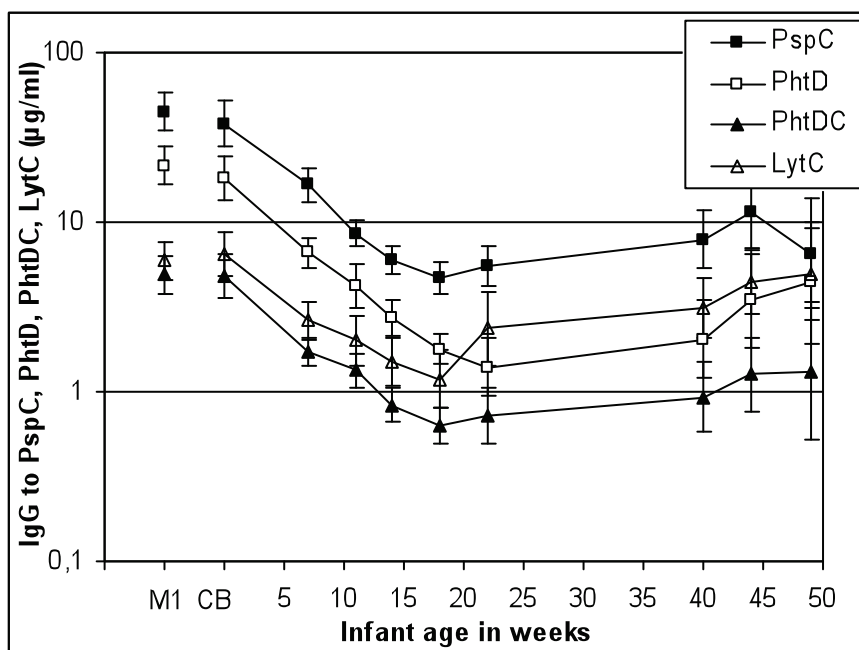


FIGURE 11. The kinetics of the development of the naturally acquired antibodies to pneumococcal proteins PspC, PhtD, PhtDC and LytC. The geometric mean concentration ($\mu\text{g/ml}$) and the 95% confidence intervals of the antibody concentrations in the sera of mothers (M1) taken during the second or third trimester of pregnancy, in the cord blood (CB) and in the consecutive sera of the infants (see Table 3). The number of samples at each time point is as follows: 52 (M1), 38–39 (CB), and for infants 42 (7 weeks), 33–34 (11 weeks), 40 (14 weeks), 29–32 (18 weeks), 31–33 (22 weeks), 40–41 (40 weeks), 27–28 (44 weeks) and 12–13 (49 weeks). Modified from (Holmlund et al. 2009).

5.5.2 Naturally acquired anti-protein antibodies in infants in relation to pneumococcal carriage and frequency of pneumococcal carriage

Frequency of pneumococcal carriage in the Filipino infants

Pneumococcal upper respiratory tract carriage is common in Filipino infants. The frequency of pneumococcal nasopharyngeal carriage of any serotype at the six study visits in the PPV1, PPV2, Control 1 and Control 2 infants is shown in Fig. 12. At 12 weeks of age, 30% of the PPV1 and PPV2 infants and 50% of the Control 1 and Control 2 infants already had been pneumococcal carriers at least once (i.e. had had a nasopharyngeal swab positive for pneumococcus). By 10 months of age (45 weeks), 80–90% of all infants had been carriers at least once (cumulative Pnc+ in Fig. 12).

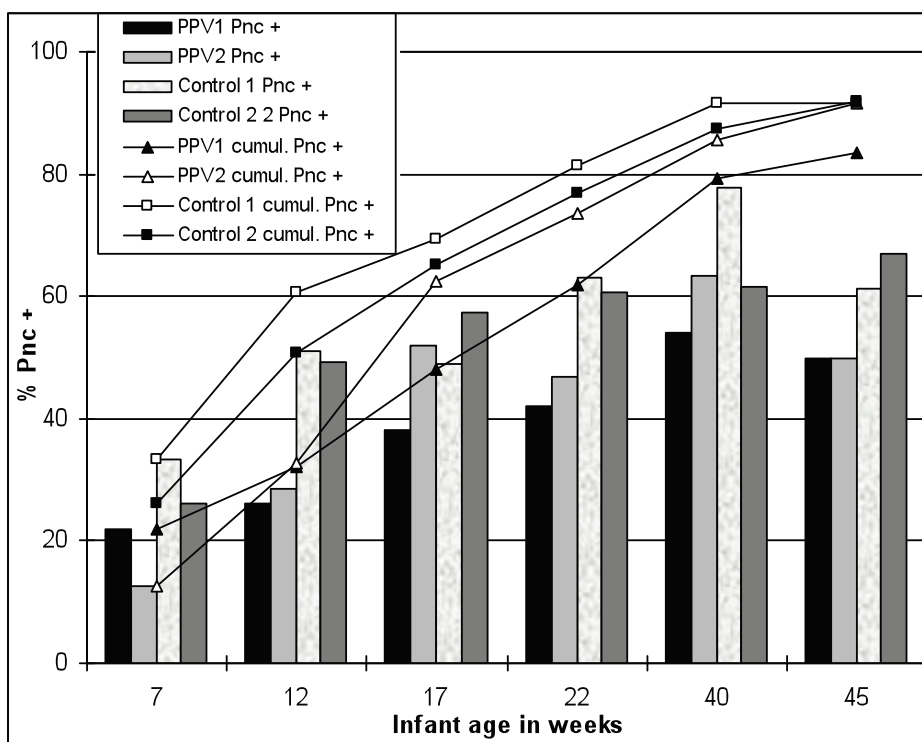


FIGURE 12. The percentage of pneumococcal carriers in the PPV1 (black bar, black triangle), PPV2 (light grey bar, white triangle), Control 1 (textured grey bar, white square) and Control 2 (dark grey bar, black square) infants at the six study visits. The bars are point estimates for pneumococcal carriage of any serotype at the visit; the line indicates the cumulative carriage of any serotype during the follow-up. The PPV1 and PPV2 results have not previously been published, but the Control 1 and Control 2 results originate from (Holmlund et al. 2006).

Using the GEE logistic marginal regression model, we found that the odds ratios to carry pneumococci between the different study groups were not time dependent ($p=0.187$), even though the cumulative carriage in the PPV2 infants quite rapidly increases between weeks 7 and 17 in Fig. 12. Using the carriage information from all time points, we show that PPV1 and PPV2 infants had significantly less pneumococcal carriage than Control 1 and Control 2 infants (Table 5). This suggests that maternal immunisation might reduce infant pneumococcal carriage, while early infant immunisation might not.

Table 5. Differences in odds ratio (OR) in carrying pneumococci in the nasopharynx in PPV1, PPV2, Control 1 and Control 2 infants. Control 2 infants are used as the reference group. CI = confidence interval. These data have not previously been published.

Group compared to Control 2 infants	OR (95% CI)
PPV1	0.52 (0.34-0.80)
PPV2	0.61 (0.41-0.89)
Control 1	1.07 (0.71-1.60)
Control 2	1

Development of naturally acquired anti-protein antibodies in relation to pneumococcal carriage (II and III)

The Control 1 and Control 2 infants were divided into two groups according to whether they had or had not culture-confirmed exposure to pneumococcus (cumulative carriage) (see section 4.2.2) (Fig. 13). Once the infant had become a carrier (i.e. had a nasopharyngeal swab positive for pneumococcus), he/she stayed in the Pnc+ group. This means that the number of infants in the carrier group (Pnc+) was cumulatively increasing and the number of infants in the Pnc- group was decreasing. In the Pnc+ group, the GMCs of the naturally acquired anti-protein antibodies increased with age, whereas the GMCs in the Pnc- group decreased with age and generally remained low (Fig. 13). The difference between the Pnc+ and Pnc- group was most clear for the anti-PsaA, -Ply and -PspC antibody concentrations, while the increases in antibody concentrations to PspA, PhtD, PhtDC and LytC were modest, but showed a similar trend. The small number of infants in the Pnc- group at the end of the follow-up resulted in wide 95% CIs.

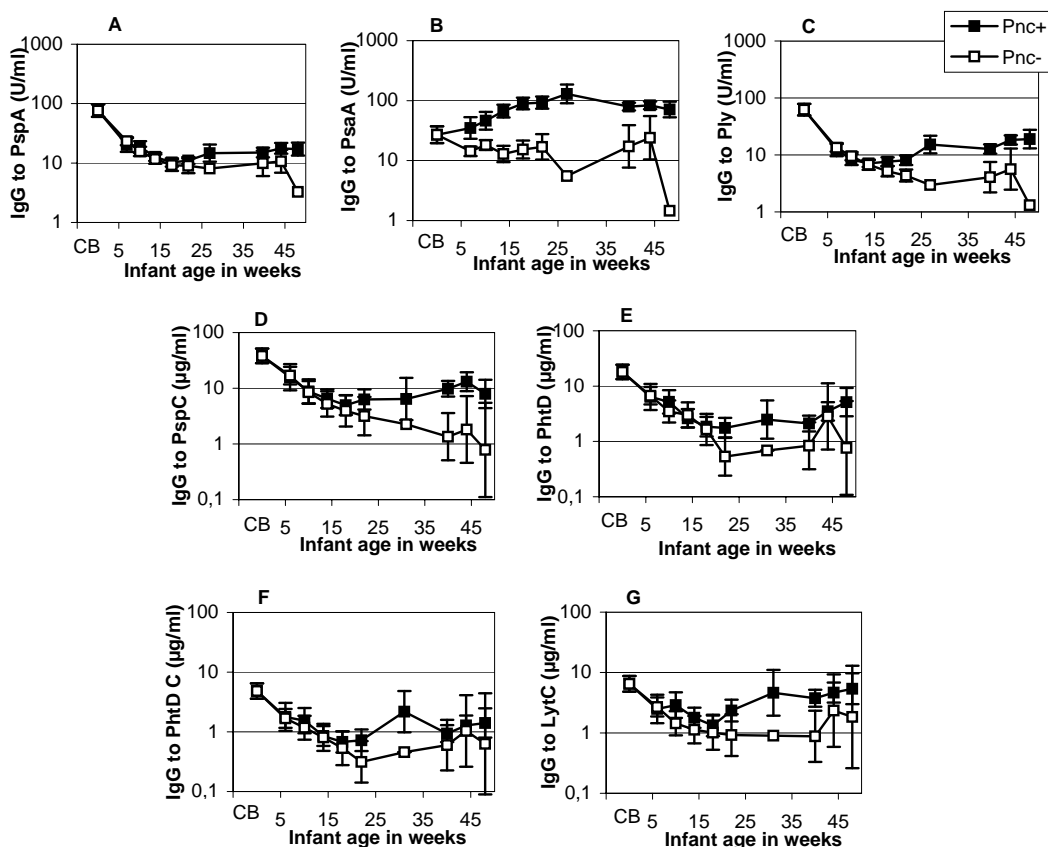


FIGURE 13. The development of naturally acquired antibodies to PspA (A), PsaA (B), Ply (C), PspC (D), PhtD (E), PhtDC (F), and LytC (G) in relation to previous or current pneumococcal nasopharyngeal carriage in the Control 1 and Control 2 infants. The geometric mean concentrations and the 95% confidence intervals of the anti-PspA, -PsaA and -Ply antibodies are expressed in U/ml, whereas the GMCs and the 95% CIs for the anti-PspC, -PhtD, -PhtDC and -LytC are expressed in µg/ml. Pnc+ = previous or current pneumococcal carrier, Pnc- = no detected pneumococcal carriage. Filled squares: Pnc+; number of samples per time point: 34 (7 weeks), 41 (10 weeks), 82 (14 weeks), 85 (18 weeks), 88–91 (22 weeks), 24 (27 weeks), 113–114 (40 weeks), 112–113 (44 weeks), 34–35 (48 weeks) (A–C) and 13 (7 weeks), 15–16 (11 weeks), 26 (14 weeks), 21–24 (18 weeks), 26–28 (22 weeks), 36–37 (40 weeks), 25–26 (44 weeks), 11–12 (49 weeks) (D–G). Open squares: Pnc-; number of samples / time point 97–98 (7 weeks), 65 (10 weeks), 54 (14 weeks), 43 (18 weeks), 25–26 (22 weeks), 1 (27 weeks), 17 (40 weeks), 12–13 (44 weeks), 1 (48 weeks) (A–C) and 29 (7 weeks), 18 (11 weeks), 14 (14 weeks), 8 (18 weeks), 5 (22 weeks), 4 (40 weeks), 2 (44 weeks) and 1 (49 weeks) (D–G). CB: cord blood; number of samples is 38–39. Modified from (Holmlund et al. 2006) and (Holmlund et al. 2009).

6 DISCUSSION

6.1 Study design

All mothers and children in this thesis were participants in the MATER study. The MATER study was a phase II trial aiming at studying the safety and immunogenicity of PPV. Pneumococcal carriage was an additional exploratory aim in preparation for a phase III study of either PPV or PCV, which took place in 2000–2004 (Lucero et al. 2009). The results of the pneumococcal carriage data will be published separately and are not included in this thesis (except for the carrier status information which was related to anti-protein antibody concentrations).

The MATER study was designed and well suited for evaluating the safety and immunogenicity of PPV in mothers; these results have been reported previously (Quiambao et al. 2003; Quiambao et al. 2007). The MATER study was also designed to evaluate the safety and immunogenicity of PPV in young infants, but not for evaluating the transfer of maternal antibodies from the mothers to the infants, since no serum samples were taken from the mothers at the time of delivery. The MATER study was neither designed for evaluating the persistence of maternal vaccine-induced antibodies in infants, because there was no study group of immunised mothers with unimmunised infants. However, since the infants did not respond to polysaccharides 6B, 14, 18C and 19F of PPV, the persistence of the maternal antibodies to these serotypes could be evaluated. A weakness of the MATER study was the lack of a relevant control group for evaluating the effect of maternal and early infant immunisation on the response to a second dose of PPV. Another weakness of the MATER study was that no saliva or breast milk samples were collected for antibody determination. A third weakness of this thesis, but not of the MATER study, is that the information on the carried serotypes was not included.

The samples of the MATER study form a very unique set of samples including the sera from the mothers, the cord blood samples and the several consecutive sera from the infants collected during the first year of life, the first samples being collected as early as seven weeks of age. The information on the pneumococcal carriage of the infants at each visit adds to the value of the sample material. The samples of the MATER study were thus utilised for the anti-protein antibody determinations. The consecutive infant sera were ideal for the determination of the natural development of anti-protein antibodies in infants and for assessing the relation between the anti-protein antibody concentrations and pneumococcal carriage. However, the long gap

(approximately five months) in sampling between visit 4 and 5 means that not all pneumococcal carriage events could be detected.

The compliance of the study infants of the MATER study was good: 84% of the 160 enrolled infants in groups PPV1, PPV2 and Control 1, and 87% of the 122 infants enrolled in Control group 2 were still participating in the study at 10 months of age. The compliance of the three-year-old toddlers was 54%, since not all toddlers could be reached and some refused the blood extraction.

6.2 Methodological issues

The EIA method was used to measure the serum antibodies of the IgG class from the study samples of pneumococcal polysaccharides of serotypes 1, 5, 6B, 14, 18C and 19F, and to pneumococcal proteins PspA, PsaA, Ply, PspC, PhtD, PhtDC and LytC. IgG was the choice, since the Ig class preferentially transferred from the mother to the foetus is IgG, and since IgG has the longest half-life in serum and is the predominant Ig class in serum (Englund 2007; Schroeder et al. 2010).

The pneumococcal polysaccharide antibody analyses were carried out in 1995–1999 using the non-22F EIA method, which was the method in common use at that time. However, the results were not published immediately and in the meantime, the EIA methodology was further developed and became more specific (Concepcion et al. 2001; Poolman et al. 2010). The more specific 22F EIA assay measures more specifically the particular anti-serotype antibody concentration in question and leaves out unspecific antibodies. This will affect the antibody concentrations, especially in unimmunised individuals, as well as the fold responses after immunisation. To find out how the improved 22F EIA method would affect the anti-polysaccharide concentrations, a subset of samples was re-analysed in 2003–2004. The sensitivity improved somewhat, as can be seen from the lower limits of quantitation of the 22F EIA. The improved sensitivity increased the number of samples with detectable concentrations, since many samples had low antibody concentrations. As expected, the specificity also improved (Concepcion et al. 2001; Soininen et al. 2002; Inostroza et al. 2005; Henckaerts et al. 2006); the concentrations detected by the 22F EIA were somewhat lower in the samples from unimmunised individuals for certain serotypes (1, 5, 6B) compared to the results obtained by non-22F EIA. Since the majority of the samples were analysed with the non-22F EIA method and the difference in results obtained by the two methods did not affect the interpretation of most of the results, the results received by the non-22F method are presented in this thesis. The only interpretation that was influenced by the choice of EIA method was that the non-22F EIA results indicated that infants

would have lower antibody concentrations after the PPV immunisation (Control 1 infants) than unimmunised infants (Control 2 infants). This could not be repeated with the 22F EIA method (Fig. 9) and the observed differences are probably due to the naturally acquired unspecific antibodies in Control 2 infants which were detected with the non-22F EIA method.

The early infant PPV immunisation was not found to affect pneumococcal carriage, since the Control 1 and 2 infants did not show any differences in the overall pneumococcal carriage rate (Fig. 12). The effect of maternal immunisation on infant pneumococcal carriage will be discussed in section 6.4.

Some of the serum samples were stored for several years before analysis (almost 10 years before the anti-PspC, -PhtD, -PhtDC and -LytC analyses were done), and some samples were thawed several times before they were analysed. Long-term storage of the serum samples is generally not thought to affect the serum IgG concentrations (Tran et al. 2006). In our laboratory, the effect of long-term storage has been tested by repeating the analyses of the first samples of a vaccine trial at a few time points during the study; the results were similar (unpublished results). We have also verified through testing that the repeated thawing and freezing of serum samples does not affect IgG antibody concentrations (unpublished).

Ideally, in addition to measuring the antibody concentrations by EIA, the functionality of the antibodies should also be measured by functional assays. The opsonophagocytic assay measures the functionality of the serum antibodies in opsonising pneumococci for phagocytosis, the most important defence mechanism against pneumococcus (Romero-Steiner et al. 1997). A good functional assay could serve as a correlate of protection, meaning that it would indicate whether or not the vaccine-induced or naturally acquired antibodies are protective against pneumococcal disease. In this study, we could have compared the functionality of vaccine-induced and naturally acquired antibodies in infant and adult sera, but unfortunately we did not have the resources for that, and currently, anti-protein antibody functional assays are scarce. To date, the functionality of anti-PspA antibodies has been shown by demonstrating that anti-PspA antibodies are able to overcome the anticomplementary effect of PspA (Ren et al. 2004; Ochs et al. 2008). For anti-PsaA antibodies, a functionality assay measuring the capacity of the anti-PsaA antibodies in inhibiting pneumococcal binding to human nasopharyngeal cells has been developed (Romero-Steiner et al. 2003). In our preliminary studies related to the functionality of anti-Ply antibodies, we have shown that infant anti-Ply antibodies induced by pneumococcal exposure are functional, i.e. they inhibit the haemolytic activity of Ply (Lindell et al. 2002). Thus, anti-Ply antibodies can be speculated to neutralise the effects of Ply in humans in a similar fashion to what has been shown in animal models (Musher et al. 2001). To develop functionality assays

that would function as correlates of protection would be very important, since large-scale clinical trials with pneumococcal protein vaccines will be difficult to conduct for ethical reasons, since four licensed pneumococcal vaccines already exist.

6.3 Protein antigen-related issues

PspA has been shown to be highly immunogenic in mice (Tart et al. 1996) and in human adults (Nabors et al. 2000). PspA is regarded as an important vaccine antigen, since anti-PspA antibodies are able to overcome the inhibition of complement deposition induced by PspA and thus increase phagocytosis of pneumococci (Ren et al. 2004). In the present study, infants were found to have quite low anti-PspA concentrations compared to adults, in accordance with previous studies (Rapola et al. 2000; Virolainen et al. 2000; Riddell et al. 2002; Laine et al. 2004). The low anti-PspA concentrations in the present study may relate to the low concentration of PspA antigen used to coat the wells of the microtiter plates (0.15 µg/ml). In the anti-PspA analyses of later studies in our laboratory, the coating concentration of PspA has been increased to 2 µg/ml, which has resulted in higher proportions of detectable anti-PspA antibodies (Simell et al. 2007; Melin et al. 2008a). In addition, antibodies to PspA were measured by using only one PspA antigen: family 1, clade 2 PspA. At the time of these analyses, the different PspA families were thought to be highly cross-protective (McDaniel et al. 1991; Tart et al. 1996; Briles et al. 2000c), whereas later, the great variations in the PspA sequence have led to the questioning of PspA's ability to induce cross-protective antibodies that would protect against PspAs representing different families and clades. It has been shown in animal studies that immunisation with a PspA protein of one family does not elicit good cross-protection against strains with PspA of another family or clade (Miyaji et al. 2002; Roche et al. 2003b). Anti-PspA in sera of human adults seems to cross-react between the PspAs of different families and clades and even between PspA and PspC (Nabors et al. 2000; Linder et al. 2007). In infants, however, the anti-PspA responses have been suggested to be rather family specific (Melin et al. 2008a). It may be that the low concentrations of anti-PspA in the infants in the present study may have resulted from catching only the PspA family 1 antibodies but not the PspA family 2 antibodies.

When the anti-PspA antibody analyses were done, the protection-eliciting epitopes were mapped to amino acid residues 1–115 and 192–260 on strain Rx1 (McDaniel et al. 1994). Thus, the truncated PspA antigen of strain Rx1 (comprising amino acids 1–315) used in the present study was supposed to contain the protection-eliciting epitopes. Recently, the proline-rich region of PspA has also been shown to be surface accessible and thereby protection eliciting (Daniels et al. 2010a). The

proline-rich region on strain Rx1 PspA comprises amino acids 288–370 (Roche et al. 2003a) and therefore the PspA fragment used in this study did not include the whole proline-rich region. This may have left some of the protection-eliciting antibodies undetected.

PsaA is a conserved pneumococcal protein that is small in size, and thus might not show on the surface due to the pneumococcal polysaccharide capsule. Despite this, production of anti-PsaA antibodies seems to start early in life, earlier in fact than any other protein antibodies looked at in the present study. The reason for the early production of anti-PsaA in infants is not known. One explanation for the increase in anti-PsaA antibody concentration could be the similar antigenic epitopes present on both pneumococcal PsaA and surface antigens of the oral streptococci known to colonise the infants at a very young age (Rotimi et al. 1981). Sequence analyses of the *psaA* gene have revealed a significant degree of homology with streptococcal putative lipoprotein adhesins (Sampson et al. 1994; Paton et al. 1997), though immunologically cross-reactive epitopes have not been found to our knowledge. PsaA could be an important vaccine antigen, since it is found on all examined pneumococcal strains, and antibodies to PsaA could inhibit the attachment of pneumococcus.

Ply is an important vaccine antigen, since it is a toxin and anti-Ply antibodies could neutralise Ply and inhibit it from causing damage to the host (Kancalerski et al. 1987; Walker et al. 1987). There are many allelic variants of PspC (Brooks-Walter et al. 1999; Iannelli et al. 2002) contributing to many different functions of pneumococci, e.g. nasopharyngeal carriage (Rosenow et al. 1997), invasion (Zhang et al. 2000) and the inhibition of complement deposition (Janulczyk et al. 2000). PspC is an important vaccine candidate, but like with PspA, a few allelic variants of PspC might be necessary to include in a future vaccine for optimal protection, although the generated antibodies might be cross-reactive between alleles at least to some degree (Brooks-Walter et al. 1999; Daniels et al. 2010a). PhtD could be an important vaccine candidate for protection against pneumococcal infections (McCool et al. 2003; Godfroid et al. 2011). PhtDC is the C-terminal end of the PhtD protein, and is thought to be the exposed and protection-eliciting end of the protein (Daniels et al. 2010a). The antibody concentrations to PhtDC were lower than to PhtD due to the smaller size of the recombinant PhtDC as compared to the full-length recombinant PhtD. LytC has been shown to promote pneumococcal colonisation (Gosink et al. 2000) and to be important in biofilm formation (Moscoso et al. 2006; Moscoso et al. 2009), for which reason anti-LytC antibodies inhibiting these functions makes LytC an important vaccine candidate. Finally, the serotype-independence of protein antigens increases their value as vaccine candidates.

6.4 Maternal antibodies

Immunisation during pregnancy is a strategy to protect neonates and young infants against bacterial or viral infections. Maternal immunisation has successfully reduced neonatal tetanus and influenza illness in infants up to six months of age (Zaman et al. 2008). The benefits of maternal immunisation for neonates and infants is thought to be mediated by the increased antibody concentrations of transferred maternal antibodies across the placenta, as well as by the increased antibody concentrations in breast milk (Munoz et al. 2001).

The data on the effect of the maternal antibodies on pneumococcal carriage are controversial. Some studies of maternal immunisation have shown that immunising the mothers with PPV reduces the frequency of pneumococcal carriage in the infant, although not statistically significantly (Munoz et al. 2001). In another study, it was shown that high maternal anti-6B antibodies might protect against carriage of serotype 6B (Auranen et al. 2002). In addition, high naturally acquired maternal anti-Ply but not anti-PspA antibody concentrations have been suggested to protect against early infant carriage (Study II; Francis et al. 2009). Other studies have failed to show that maternal immunisation with PPV (Lopes et al. 2009) or naturally acquired anti-protein antibodies (Rapola et al. 2003; Adrian et al. 2004; Simell et al. 2006; Lebon et al. 2011) would reduce pneumococcal carriage in infants. These differences may originate from variations in the ages of the study infants, the low number of pneumococcal carriers in some of the studies and differences in methodology. In this thesis, we show that the PPV1 and PPV2 infants of vaccinated mothers had less pneumococcal carriage compared to the Control 1 and Control 2 infants of unvaccinated mothers, which indicates that maternal immunisation might reduce infant pneumococcal carriage. The reduction in carriage could be due to protective maternal antibodies, but it is not likely that the reduced maternal carriage would explain the reduced carriage of the infants, since the reduced transmission of pneumococci from the mothers to the infants has been shown to have only a minimal effect on infant carriage (Darboe et al. 2010). The effect of the transferred maternal antibodies on the infants' pneumococcal carriage cannot be clarified in this thesis, since we did not examine which serotype the infant carried.

6.4.1 Transfer of maternal antibodies to the foetus

In the MATER study, maternal anti-polysaccharide antibodies were found to be transferred from the mothers to the infants (Quiambao et al. 2007). Since the maternal antibody concentrations were not measured right before birth, the antibody concentrations upon enrolment (in the second to third trimester of pregnancy) for Control 1 mothers and the post-vaccination antibody concentrations for the PPV

mothers were compared to the antibody concentrations of the cord blood samples. The transmission of anti-6B and anti-14 IgG was 57–71% (maternal antibody concentration one month post-vaccination compared to the cord blood concentration) in the PPV-vaccinated mothers and 52–88% (maternal antibody concentration upon enrolment compared to the cord blood concentration) in the mothers of the Control 1 group (Quiambao et al. 2007). We have shown that IgG antibodies to pneumococcal proteins PspA, PsaA, Ply, PspC, PhtD and LytC were also transferred from the mothers to the infants. The transmission (maternal antibody concentration upon enrolment compared to the cord blood concentration) of anti-protein IgG was approximately 100%.

Our results are in accordance with the results of previous studies. Several studies have shown that less polysaccharide IgG antibodies than protein IgG antibodies are transferred from the mothers to the infants (Shahid et al. 1995; Munoz et al. 2001; Baril et al. 2004a; Katsurahara et al. 2008; van den Berg et al. 2010). Transfer of maternal antibodies of the IgG1 subclass has been shown to be significantly more efficient than that of the IgG2 subclass (Garty et al. 1994; Malek et al. 1996). Human adults vaccinated with polysaccharides display an IgG2-predominant distribution, whereas the anti-protein antibodies are predominantly of the IgG1 subclass (Einhorn et al. 1987; Soininen et al. 1999; Munoz et al. 2001; Baril et al. 2004a). This could explain why the ratio of transferred protein antibodies is larger than that of polysaccharide antibodies.

6.4.2 Persistence of maternal antibodies in the infant

The higher the concentrations of serotype-specific IgG antibodies the newborns have received from their mothers, the longer the protection can be assumed to persist after birth. Vaccinating the mothers of this study during pregnancy gave the newborn infants significantly higher serum anti-polysaccharide antibody concentrations for the first 4–5 months of life as compared to the infants of the unvaccinated mothers. These results do not have direct implications for infants that have not been vaccinated though, since the vaccinations of the infants might have had some effects on the antibody concentrations which could not be indicated, since all infants were immunised. In other studies, similar persistence of maternal anti-polysaccharide antibodies has been reported as in this study: Maternal pneumococcal anti-polysaccharide antibodies are suggested to persist in infants for approximately 2–6 months, depending on the serotype and the study design (Shahid et al. 1995; O'Dempsey et al. 1996; Munoz et al. 2001; Lehmann et al. 2002; Almeida et al. 2009).

Since the infants started their own anti-protein antibody production very early, this will have an effect on the examination of the persistence of the maternal anti-protein antibodies. In this study, we estimate that the maternal anti-protein antibodies persisted for approximately 4–5 months after birth. Other studies have suggested that the maternal anti-protein antibodies persist for 5–6 months (Zhang et al. 2006a; Lebon et al. 2011).

6.4.3 Effect of high maternal anti-polysaccharide antibody concentration on the response of infants to PPV

Only a few of the PPV1 infants with high maternal anti-polysaccharide antibody concentrations had detectable increases in antibody concentration after immunisation with PPV, which is in concordance with earlier reports (Klein et al.; Nohynek et al. 1999; Siegrist 2003). In a mouse model, high levels of maternal anti-polysaccharide antibodies completely inhibited the responses of the young mice to the polysaccharide, but low or moderate levels of maternal antibodies did not interfere with, or even enhanced, the immune response of the offspring (Richter et al. 2005).

When analysing the individual responses of the infants of the MATER study, we found that at seven weeks of age, the infants with an antibody concentration higher than $\sim 3 \mu\text{g/ml}$ did not generally respond to the PPV vaccination (data not shown). The fact that an infant with a high maternal antibody concentration did not respond to PPV does not mean that the protection would be inferior, but rather indicates that the antibody concentration has already reached a high concentration and no higher concentrations could be expected due to the normal immunological regulation. In addition, when examining the responses of the infants at an early age, we need to remember that the post-concentration is compared against a changing background, since the maternal antibodies decrease by age.

6.5 PPV-induced and naturally acquired anti-polysaccharide antibodies in infants

Pneumococcal polysaccharide vaccines are regarded as poorly immunogenic in infants and children less than two years of age (Sell et al. 1981; Douglas et al. 1983; Koskela et al. 1986; Temple et al. 1991). We have shown that infants at seven weeks of age are able to respond to pneumococcal serotypes 1 and 5, but not to serotypes 6B, 14, 18C and 19F. In accordance with this, it has been shown previously, though not in infants this young, that not all polysaccharides are poor immunogens in young

children. Infants younger than two years have been shown to respond to certain serotypes in PPV, such as 1, 2, 3, 5, 7F and 23F (Sell et al. 1981; Douglas et al. 1983; Koskela et al. 1986; Temple et al. 1991; Pomat et al. 1994). In a recent immunisation study in Fiji, most of the 23 serotypes of PPV were shown to be immunogenic in children aged 12 months (Balloch et al. 2010). The least immunogenic serotypes in the Fijian study were shown to be 6B, 14, 19F and 23F, which is in accordance with our results. In contrast to our study, serotype 18C was found to be immunogenic both in Finnish and Fijian infants (Koskela et al. 1986; Balloch et al. 2010). The discrepancy with the immunogenicity of 18C in young children may be related to differences in the ages of the children studied.

The kinetics of the vaccine-induced and naturally acquired anti-polysaccharide antibody concentrations from birth to 10 months of age was evaluated in the present study. In unimmunised infants, the concentrations decreased as the maternal antibodies disappeared and remained low until 10 months of age. In the PPV-immunised children, the kinetic curves to polysaccharides 6B, 14, 18C and 19F were similar to the kinetic curves of the unimmunised infants, with one exception: the concentrations were higher for the first 4–5 months, since the mothers of the PPV infants had been immunised. For serotypes 1 and 5, the concentrations increased in response to PPV, but decreased again after that. In general, concentrations of anti-polysaccharide antibodies induced by polysaccharide vaccines in young children start to decrease soon after the vaccine-induced response (Sell et al. 1981; Koskela et al. 1986). In our study, this was true for serotype 5, but the decrease to serotype 1 was much slower. The PPV-immunised infants had statistically significantly higher antibody concentrations to serotype 1 until the age of nine months as compared to the unimmunised infants. Serotype 1 is a zwitterionic polysaccharide, meaning that the polysaccharide is able to elicit T-cell responses even in the absence of a conjugated protein carrier (Kalka-Moll et al. 2002; Velez et al. 2009). Immunising mice with pneumococcal serotype 1 resulted in an increase in serum anti-1 IgG and the existence of a humoral memory response was suggested (Kalka-Moll et al. 2002; Groneck et al. 2009). Serotype 1 is a common serotype causing IPD in the Philippines (Lupisan et al. 2000). Thus, the frequent occurrence of this serotype might have induced natural boosting in the Filipino infants. However, serotype 1 carriage is rarely detected, since the duration of the carriage is usually very short (Sleeman et al. 2006). This raises the question of whether the very short duration of carriage could keep the concentrations elevated in the infants.

In Papua New Guinean children less than two years of age, the 14- and 23-valent pneumococcal polysaccharide vaccines have been shown to protect against death caused by acute lower respiratory tract infections (Riley et al. 1986; Lehmann et al. 1991). The authors point out that in low-income countries, children are exposed to invasive serotypes which might evoke good immune responses relatively early in

life. According to this thesis, serotypes 1 and 5 are immunogenic serotypes early in life, and since they are common serotypes in Papua New Guinea (Barker et al. 1989), it can be hypothesised that serotypes 1 and 5, as well as other immunogenic serotypes not evaluated in this thesis, protected the children against death.

6.5.1 Response to a second dose of PPV at toddler age

Most pathogens enter our body via mucosal sites, which will induce efficient immune responses. At the same time, the commensal bacteria and food antigens have to be recognised without eliciting an active immune response. The immune system also has to stay unresponsive to self antigens. Immunising women during pregnancy and infants early in life, when the immune defence of the infant is still not fully developed, has raised the question of whether the development of the infants' immune system will be disturbed and become unresponsive to the vaccine antigens and later also to the pathogens.

Hyporesponsiveness to the polysaccharide vaccines, especially in young children, has been a topic of extensive debate over the past 20 years. Conflicting data have been reported by different studies; some showed hyporesponsiveness to a second dose of a polysaccharide vaccine, while others did not (Borgoño et al. 1978; Douglas et al. 1983; Lawrence et al. 1983; Koskela et al. 1986; MacLennan et al. 1999; Russell et al. 2010). Recently, it has been demonstrated that nasopharyngeal carriage of pneumococcus at the time of immunisation with PCV causes serotype-specific hyporesponsiveness (Dagan et al. 2010; Väkeväinen et al. 2010). The immune mechanisms responsible for the development of immune hyporesponsiveness are not known, but one explanation suggested is the depletion of the B-cells by the T-cell-independent polysaccharides in the vaccine. In a study by Lazarus and co-workers, decreases in serotype-specific memory B-cells were shown in adults after immunisation with PPV (Lazarus et al. 2010). In other studies, it has been suggested that primary immunisation with polysaccharides induces B-cells to become antibody-producing plasma cells, but not memory cells (Granoff et al. 2007; Poolman et al. 2011). Thus, the B-cell pool will be consumed, resulting in a hampered antibody production after the second dose. This could explain the findings by O'Grady and co-workers (O'Grady et al. 2010) that PPV in particular increased the risk of hospitalisation for acute lower respiratory tract infections among Australian indigenous infants 5–23 months of age. The hyporesponsiveness issue is an important aspect to consider when planning vaccination programs in early infancy and clearly needs further investigation.

In the present study, the mothers were vaccinated with PPV during pregnancy and the infants were vaccinated at a very young age. Approximately three years after the primary vaccination, some of the study children received a second dose of PPV. The antibody response to the second dose of PPV was good in the PPV1, PPV2 and Control 1 toddlers, indicating that the maternal and/or early infant immunisations did not induce hyporesponsiveness to PPV in infants. However, it is possible that the good response of the toddlers was due to the long interval between the first and the second dose: the children had had time to produce a new pool of B-cells and the antibody concentrations regulating the responses were low.

6.5.2 Vaccine-induced anti-polysaccharide antibodies and protection against pneumococcal infection

The anti-capsular antibodies are the major mechanism by which the polysaccharide vaccines provide protection against pneumococci (Denoël et al. 2011). Based on clinical efficacy studies of PCVs in infants, a level of IgG antibodies of 0.35 µg/ml has been estimated to be associated with protection against IPD at a population-based level (WHO 2009). In the case of mucosal pneumococcal infections, such as pneumococcal pneumonia, AOM and pneumococcal carriage, the antibody concentrations required for potential protection are probably higher. It has been suggested that a concentration of >1 µg/ml might be associated with protection against pneumonia in the elderly (Örtqvist et al. 2007). In adults, a concentration of ≥5 µg/ml of anti-14 IgG antibodies has been shown to be associated with protection against pneumococcal carriage of serotype 14 (Goldblatt et al. 2005). However, it should be noted that the protection offered by antibodies probably varies between different pneumococcal serotypes and distinct thresholds for each serotype would be required to better describe the antibody-mediated protection. The antibody concentration required for antibody-mediated protection may also vary with age and with the amount of pneumococcal exposure.

In the MATER study, the clinical outcome of the vaccination was not examined; the aim was to assess immunogenicity. Thus, the association with protection had to be evaluated through the antibody concentrations. In this thesis, the proportions of infants with anti-polysaccharide antibody concentrations higher than 0.35 µg/ml or 1 µg/ml during the first year of life were evaluated (Fig. 8). At seven weeks of age, the proportion of PPV1 and PPV2 infants with antibody concentrations to the six serotypes above the two thresholds was 60–100%, due to the high concentration of maternal antibodies. Among the Control 1 infants, the proportion of infants with antibody concentrations higher than 0.35 µg/ml or 1 µg/ml varied between 40–80% and 10–60%, respectively. Immunisation of the infants with PPV increased the

proportion of infants with antibody concentrations to serotypes 1 and 5 that exceeded the thresholds, after which the proportions started to decrease again. At 10 months of age, very few infants had antibody concentrations to 6B and 18C higher than the thresholds, indicating that they might be susceptible to pneumococcal disease. In accordance with this, the high incidence of pneumonia and pneumococcal carriage in infants in low-income countries (Rudan et al. 2008) could be due to the lack of protective immunity after the maternal antibodies have disappeared (Fig. 8). In this study, the percentage of three year olds with concentrations above 0.35 µg/ml varied greatly, from 0–57% in the pre-immunisation samples to 86–100% in the majority of the post-immunisation samples (Table 4). The corresponding percentages of concentrations >1 µg/ml were lower. This indicates that after the second dose of PPV, the majority of the infants had concentrations associated with protection against IPD.

6.6 Naturally acquired serum anti-protein antibodies in infants

Naturally acquired IgG antibodies to pneumococcal proteins PspA, PsaA, Ply, PspC, PhtD, PhtDC and LytC were shown to be produced in infants (Fig. 10 and 11). The development of anti-PsaA differs from the development of antibodies to the other pneumococcal proteins (Rapola et al. 2000; Zhang et al. 2006a; Lebon et al. 2011). Accordingly, increases in the antibody concentrations to PsaA in the Filipino infants were already detected after seven weeks of age, indicating that the anti-PsaA production of infants starts at an early age. In British infants (Zhang et al. 2006a), the naturally induced anti-PsaA antibody production was shown to start after two months of age, whereas in Finnish (Rapola et al. 2000) and Dutch infants (Lebon et al. 2011), the anti-PsaA concentrations did not decrease at all after birth, most likely because the first sample from the infant was not taken before six months of age. The differences observed in the onset of anti-PsaA production are probably due to the study designs, but they all show the exceptionally early onset of antibody production to PsaA compared with the other protein antigens.

The production of antibodies to the other pneumococcal proteins (PspA, Ply, PspC, PhtD, PhtDC and LytC) started after 4–5 months of age in Filipino children (Fig. 10 and 11). In Dutch children, the production of anti-protein antibodies to several proteins, e.g. PsaA, PspC, PhtD and Ply, started after six months of age (Lebon et al. 2011). In British children, the anti-PspA, anti-Ply and anti-PspC antibody production did not start until after 13 months of age (Zhang et al. 2006a). In Finnish children, the antibody production to PspA, Ply, PpmA, PhtB, PhtE, NanA, PspC and PhtD has been shown to start after 12 months of age (Rapola et al. 2000; Bogaert et al. 2006; Simell et al. 2006; Holmlund et al. 2007; Simell et al. 2009).

Since adults generally have much fewer pneumococcal infections than children, it can be speculated that the adult concentration of antibodies could be a threshold concentration associated with protection against pneumococcal disease. The anti-PsaA concentration of the Filipino, Finnish and Kenyan infants have been shown to reach the adult concentration at 14 weeks, 6 months and <1 year of age, respectively (Rapola et al. 2000; Laine et al. 2004). The anti-LytC and anti-NanA concentration of the Filipino and Finnish children reached the concentration of adults at 11 months and 2 years of age, respectively (Simell et al. 2006), whereas the antibody concentrations to the other proteins included in this thesis (i.e. PspA, Ply, PspC, PhtD) have been shown to remain below the adult concentrations until two years of age (Rapola et al. 2000; Bogaert et al. 2006; Simell et al. 2006; Holmlund et al. 2007). The early onset of antibody production to PsaA, LytC and NanA clearly shows that these proteins are immunogenic in young infants and children. The early onset of the anti-PsaA, -LytC and -NanA antibody production could be due to similar cross-reacting proteins found on other streptococcal species (Hakenbeck et al. 2009; Zhang et al. 2010; Löfling et al. 2011).

6.6.1 The association of naturally acquired anti-protein antibody concentrations and pneumococcal carriage

Pneumococcal carriage is frequent among Filipino infants less than one year of age (Fig. 12). Naturally acquired serum and mucosal immunity against pneumococcus is suggested to develop by pneumococcal contacts (Rapola et al. 2000; Simell et al. 2001; Soininen et al. 2001; Simell et al. 2002), and antibodies to both capsular polysaccharides and proteins are thought to play a role in prevention of pneumococcal infection (Black et al. 2000; Rapola et al. 2001b). In the present study, children with previous or current pneumococcal colonisation had higher antibody concentrations to pneumococcal proteins PspA, PsaA, Ply, PspC, PhtD, PhtDC and LytC than the non-colonised children (Fig. 13). The difference in the antibody concentrations between the carriers and non-carriers could be seen early, at seven weeks of age for anti-PsaA and somewhat later for the other proteins. For the anti-PspA concentrations, the difference between the carriers and non-carriers was not that clear cut, probably because the overall responses to PspA were found to be quite poor in our analyses. Our results confirmed that the natural development of antibodies to PspA, PsaA, Ply, PspC, PhtD, PhtDC and LytC is associated with pneumococcal carriage (Fig. 13). The role of pneumococcal exposure in induction of the production of naturally induced serum anti-protein antibodies has also been shown earlier in Finnish and Dutch children younger than two years of age (Rapola et al. 2000; Bogaert et al. 2006; Simell et al. 2006; Holmlund et al. 2007; Simell et

al. 2009; Lebon et al. 2011). In contrast, British children 2–12 years of age colonised with pneumococcus have been shown to have lower serum anti-PspC and anti-Ply concentrations at the time of swabbing as compared to the non-colonised children (Zhang et al. 2006a). In addition, Gambian infants 3–5 months of age colonised with pneumococcus had lower anti-PsaA concentrations as compared to infants with cultures negative for pneumococcus (Obaro et al. 2000). This indicates that a lack of specific antibody to protein determinants may correlate with a risk of colonisation (Obaro et al. 2000; Zhang et al. 2006a). The reason for the different results is not known.

6.6.2 The naturally acquired anti-protein antibodies and protection against pneumococcal infection

The role of naturally acquired anti-protein antibodies in protection against pneumococcal infections has been evaluated in animals and humans. Pneumococcal colonisation induces an increase in anti-protein antibodies, as has been shown in this study and by others (Lebon et al. 2011). In a human colonisation model, anti-PspC and anti-PspA antibodies were suggested to have a role in prevention of pneumococcal colonisation (McCool et al. 2002). In contrast to this, it has been shown in children older than one year of age that a higher serum anti-PsaA concentration is associated with an increased risk of pneumococcal carriage, whereas the risk of pneumococcal AOM decreases (Rapola et al. 2003). In accordance with this, it has been shown that a higher concentration of naturally acquired anti-protein antibodies to PhtE, NanA, PpmA, PsaA, SlrA, SP0189 and SP1003 at 6 and/or 14 months of age was not associated with protection against pneumococcal colonisation, but was associated with a decreased number of respiratory tract infections in the third year of life (Lebon et al. 2011). In addition, naturally acquired human anti-PhtD antibodies transferred to mice have shown significant protection against pneumococcal infection (Godfroid et al. 2011).

In the light of the knowledge we have today, the protection against pneumococcal infections is a complex issue. In addition to systemic antibody-mediated immunity, mucosal- and T-cell-mediated immunity is also important. Salivary anti-PspA antibodies in children have not been associated with protection against pneumococcal carriage, but have been shown to be associated with a lowered risk of pneumococcal AOM (Simell et al. 2007). In a mouse model, protection against pneumococcal carriage has been shown to be dependent on the CD4⁺ T-cells and independent of pneumococcal antibodies (Trzcinski et al. 2005). Cytokines IFN- γ and IL-10 have been suggested to regulate the production of mucosal anti-Ply and anti-PspC antibodies (Zhang et al. 2006b). In addition, IL-17A secreted by CD4⁺ T-cells has been shown to mediate immunity against pneumococcal carriage (Lu et al.

2008). This was recently confirmed by the two novel protein antigens, SP2108 and SP0148, that stimulated the IL-17A secreting CD4⁺ T-cells in mice and provided protection against pneumococcal colonisation (Moffitt et al. 2011). In conclusion; naturally acquired anti-protein antibodies might not be directly or solely important in the protection against pneumococcal colonisation in children, but they might be associated with protection against AOM and respiratory tract infections.

7 CONCLUSIONS

The following conclusions were made on the basis of the studies in this thesis:

1. The infants of the PPV immunised mothers had significantly higher antibody concentrations to serotypes 6B, 14, 18C and 19F during the first 4–5 months of life as compared to the infants of unimmunised mothers. This indicates that maternal immunisation with PPV might extend the protection of the infant against pneumococcal infection.
2. Infants were indeed able to respond to some pneumococcal serotypes, like 1 and 5, after immunisation with PPV at 7 or 17 weeks of age. However, they were not able to respond to many clinically relevant serotypes like 6B, 14, 18C and 19F at this early age. Today, PPV should not be used at this early age, since the conjugate vaccines provide better protection.
3. A high maternal anti-polysaccharide antibody concentration at seven weeks of age inhibited or reduced the responses of the infants to PPV. Despite the inhibition, the concentrations in the non-responders were not inferior compared to the responders (with lower maternal antibody concentrations), suggesting that the protection in the non-responders was not inferior.
4. After the maternal antibodies had disappeared, the antibody concentrations to serotypes 1, 5, 6B, 14, 18C and 19F of the unimmunised infants remained low until the age of 10 months.
5. At three years of age, the toddlers responded well to a second dose of PPV, suggesting that maternal and/or early infant immunisation might not interfere with the antibody responses to re-immunisations.
6. The 22F EIA and the non-22F EIA methods gave similar concentrations in the sera of immunised infants, but in unimmunised infants the non-22F EIA gave higher concentrations. Thus, the fold increases in response to PPV and antibody concentrations of the infants and toddlers of this study should not be compared to other studies where the results have been obtained by the 22F EIA method.
7. Naturally acquired antibodies to pneumococcal proteins PspA, PsaA, Ply, PspC, PhtD, PhtDC and LytC were transferred from the mother to the infant;

this might provide the newborn child with passive protection against pneumococcal disease.

8. The infants started their own production of naturally induced antibodies to PspA, Ply, PspC, PhtD, PhtDC and LytC at 4–5 months of age, and already after seven weeks of age to PsaA.
9. Since the infants started their own naturally induced anti-protein antibody production very early, this will have an effect on the examination of the persistence of the maternal anti-protein antibodies. Thus, the persistence of the maternal anti-PsaA antibodies in the infants could not be determined. The persistence of the maternal antibodies to PspA, Ply, PspC, PhtD, PhtDC and LytC was estimated to persist in the infant for approximately 4–5 months.
10. Nasopharyngeal culture–confirmed pneumococcal carriage clearly induced the production of antibodies to the seven protein antigens analysed in this thesis.

8 FUTURE CONSIDERATIONS

Every year, pneumococcal infections kill up to one million children, particularly children living in low-income countries. The best way to protect the very young against pneumococcal disease could be maternal immunisation. By immunising the mother, the infant seems to get significantly higher maternal antibody concentrations through the placenta and breast milk, which will extend the time of antibody-mediated protection in the infant. The longer protection offered by maternal antibodies is especially needed in low-income countries, since in these settings the immunisations of the infants may be delayed.

PCVs have been successfully introduced to infants in many industrialised countries and through a Global Alliance for Vaccines and Immunization (GAVI) initiative, as well in a few low-income countries. They have been shown to protect against disease caused by the vaccine serotypes, and they have also reduced pneumococcal carriage by the vaccine serotypes. There is, however, some concern about the use of PCVs in high-risk populations in low-income countries. In low-income countries, a broader number of serotypes cause diseases and death, thereby reducing the overall impact of PCVs. There may also be a greater risk of serotype replacement in pneumococcal disease. PPV has a broader coverage of serotypes, but is not immunogenic against several important pneumococcal serotypes in infants.

The pneumococcal proteins might be used as alternative carrier proteins in the PCVs. A pneumococcal protein used as a carrier would not induce interfering antibodies with other vaccines (e.g. diphtheria and tetanus vaccines) and any immunity against the pneumococcal carrier protein would add to the protective effect of the polysaccharide, thereby resulting in a broader overall protection. A PspA protein conjugated to the capsular polysaccharides of serotypes 1 or 5 has been shown to induce higher survival rates in mice as compared to mice immunised with PspA or polysaccharides alone. In addition, Ply has been used in experimental PCVs, which have been more immunogenic and protective in mouse models as compared to the polysaccharide or Ply alone. Since the PCVs are expensive to manufacture, however, they are not the ideal choice for low-income countries.

Due to the problems related to the polysaccharide-based vaccines, vaccines based on common pneumococcal protein antigens would be a good alternative for immunisation against pneumococci, especially in low-income countries. The protein vaccines would provide protection regardless of the serotype and the costs of their manufacture are expected to be lower compared to that of the PCVs. In animal

models, immunisations with pneumococcal proteins PspA, PsaA, Ply, PspC, PhtD and LytC have been shown to induce protection against pneumococcal carriage and disease. The protein-based pneumococcal vaccines are now in the early stages of clinical research, but their licensing will be problematic: Large-scale efficacy trials will be very difficult to conduct for ethical reasons, since four pneumococcal vaccines are already licensed and used in national vaccination programs in many countries. To be able to license pneumococcal protein vaccines, laboratory correlates of protection should be developed. To be able to develop such laboratory methods, the virulence mechanisms of the proteins and how immunity to them is generated, as well as how the immune response should be measured needs to be further investigated.

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